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Wheat amino acid transporters highly expressed in grain cells regulate grain size and grain nitrogen accumulation

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2 **Wheat amino acid transporters highly expressed in grain cells regulate grain size**
3 **and grain nitrogen accumulation**

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Abstract: Amino acids are delivered into developing wheat grains to support the accumulation of storage proteins in the starchy endosperm, and transporters play important roles in regulating this process. RNA-seq, RT-qPCR, and promoter-GUS assays showed that three amino acid transporters (TaAAP2, TaAAP13, and TaAAP21) are differentially expressed in the endosperm transfer cells, starchy endosperm cells, aleurone cells and the embryo of the developing grain, respectively. Yeast complementation revealed that all three transporters can transport a broad spectrum of amino acids. RNAi-mediated suppression of TaAAP13 expression in the starchy endosperm did not reduce the total nitrogen content of the whole grain, but significantly altered the composition and distribution of metabolites in the starchy endosperm, with increasing concentrations of some amino acids (especially glutamine and glycine) from the outer to inner starchy endosperm cells. Overexpression of TaAAP13 under the endosperm-specific HMW-GS promoter significantly increased grain size, grain nitrogen concentration, and thousand grain weight, indicating that the sink strength for nitrogen improvement was increased by manipulation of amino acid transporters. However, the total grain number was reduced, suggesting that source nitrogen remobilized from leaves is a limiting factor for productivity. Therefore, simultaneously increasing loading of amino acids into the phloem and delivery to the spike would be required to increase protein content while maintaining grain yield.

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Keywords: amino acid transporter, wheat grain, overexpression, RNAi, grain nitrogen, grain metabolites, grain size.

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Abbreviation: alcohol dehydrogenase (ADH), aleurone cell (AL), amino acid/auxin permease subfamily (AAP), amino acid permease (AAP), amino acid polyamine-choline transporters subfamily (APC), amino acid transporter (AAT), analysis of variance (ANOVA), cetyl trimethylammonium bromide (CTAB), complementary DNA (cDNA), days post anthesis (DPA), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), drug/metabolite transporter (DMT), ethylenediaminetetraacetic acid (EDTA), endosperm transfer cell (ETC), frequency per kilobase million (FPKM), genetically modified (GM), β -glucuronidase (GUS), grain protein content (GPC), high molecular weight glutenin subunits (HMW-GS), high performance liquid chromatography (HPLC), ^1H -Nuclear magnetic resonance (NMR), Isoamyl alcohol (IAA), least significant difference (LSD), Nucellar projection transfer cell (NPTC), orthogonal projections to latent structures discriminant analysis (OPLS-DA), partial least squares discriminant analysis (PLS-DA), plasma membrane H^+ - ATPase (PMA), principal component analysis (PCA), protein body (PB), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), RNA interference (RNAi), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), starchy endosperm (SE), starch granule (SG), thousand grain weight (TGW), trichloroacetic acid (TCA), usually multiple amino acids move in and out transporter (UMAMIT).

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67 Introduction

68 Wheat contributes about 10% of the dietary intake of protein in the UK, and grain protein
69 content (GPC) is a key determinant of breadmaking quality. Farmers routinely increase
70 the grain protein content by applying more inorganic nitrogen fertilisers, which increases
71 the cost of production and may have an adverse environmental footprint. Therefore,
72 improved nitrogen use efficiency is a long-term strategy for sustainable improvement of
73 wheat productivity and grain protein content.

74 Amino acids are the major transported form of reduced nitrogen in the plant (Palmer *et al.*,
75 2014; Riens *et al.*, 1991). The transport of amino acids across membranes and
76 translocation from source to sink is mediated by membrane transport proteins: amino acid
77 transporters (AATs). These are classified into two major subfamilies: the Amino
78 Acid/Auxin Permease family (AAP) and the Amino acid-Polyamine-Choline transporters
79 family (APC) (Lee and Tegeder, 2004; Rentsch *et al.*, 2007). In addition, a new group,
80 the Usually Multiple Amino Acids Move In and Out Transporter (UMAMIT) family, has
81 been identified (Denancé *et al.*, 2010), which is part of the Drug/Metabolite Transporter
82 (DMT) family (Jack *et al.*, 2001). More than 110 AAT genes have been identified in
83 Arabidopsis and genome-wide surveys of amino acid transporters have been reported for
84 many plant species including rice (Zhao *et al.*, 2012), poplar (Wu *et al.*, 2015), potato (Ma
85 *et al.*, 2016) and wheat (Wan *et al.*, 2017).

86 Only a few AAT genes have been functionally characterized, mainly in Arabidopsis.
87 Nevertheless, studies indicate that they play important roles in amino acid uptake into
88 roots, phloem loading, long-distance transport and loading into the seed (Dinkeloo *et al.*,
89 2017; Tegeder, 2012). Crucial functions for the import of amino acids into sink tissues
90 (seed, fruit, and tuber) have been described for different amino acid transporters. The
91 AtAAP1 transporter is highly expressed in the embryo epithelium (transfer cells) of
92 Arabidopsis and involved in uptake and transport of amino acids from the endosperm into
93 the embryo, with *ataap1* mutants having lower seed protein content and seed weight
94 (Sanders *et al.*, 2009). RNAi-mediated down-regulation of the StAAP1 transporter can
95 reduce the levels of free amino acids by 50% in potato tubers (Koch *et al.*, 2003). The
96 tonoplast-localized UMAMIT24 of Arabidopsis is able to transport the amino acids
97 temporarily stored in the vacuoles of chalaza cells before being delivered to filial tissue,
98 while the plasma membrane-localized UMAMIT 25 is expressed in the endosperm and
99 pericarp and could mediate amino acid export from endosperm (Besnard *et al.*, 2018).
100 The UMAMIT18 transporter is present in vascular tissues and developing seeds,
101 supporting accumulation nitrogen in developing siliques (Ladwig *et al.*, 2012). UMAMIT11
102 and 14 AATs are expressed in the chalaza (unloading domain) of developing seeds and
103 UMANIT28 and 29 transporters export amino acids from the endosperm and pericarp,
104 respectively. Single loss-of-function mutants of these four transporters resulted in the
105 accumulation of high levels of free amino acids in the seeds and greatly reduced seed
106 size (Müller *et al.*, 2015). By contrast, overexpression of VfAAP1 (which is highly
107 expressed in embryonic storage parenchyma cells at early maturation) in both pea and

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3 108 *Vicia narbonensis* (Narbon bean) seeds resulted in increased seed protein content (by
4 109 10-25%) and seed size (by 20-30%), by increasing import of amino acids into the embryo
5 110 (Rolletschek *et al.*, 2005). Similarly, the simultaneous overexpression of PsAAP1 in the
6 111 phloem and embryos of pea plants increased seed yield and seed storage protein levels
7 112 when the plants were grown with highly abundant N, due to increased source-to-sink
8 113 allocation of amino acids (Zhang *et al.*, 2015) and increased nitrogen use efficiency
9 114 (Perchlik and Tegeder, 2017). The OsAAP6 transporter, which is highly expressed in the
10 115 endosperm of rice, functions as a positive regulator of GPC and overexpression in rice
11 116 greatly increased GPC in rice grain when overexpressed (Peng *et al.*, 2014). Tomato
12 117 SICAT9 is a tonoplast Glu/Asp/GABA transporter, and overexpression of SICAT9 greatly
13 118 affects the flavor profile of the tomato fruit by increasing the accumulation of these amino
14 119 acids during fruit development (Snowden *et al.*, 2015).

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19 120 Wheat grains comprise three distinct parts, the embryo, the outer layers (nucellar
20 121 epidermis, testa and pericarp), and the endosperm, which account for about 3%, 7%, and
21 122 90% of the grain weight, respectively (Barron *et al.*, 2007). The endosperm consists of
22 123 three cell types, endosperm transfer cells (ETC), aleurone cells (AL) and starchy
23 124 endosperm cells (SE). Amino acids are unloaded from the phloem via the vascular bundle
24 125 into the endosperm cavity of the grain (Wang *et al.*, 1994b), where they are actively taken
25 126 up by transfer cells; this is the first bottleneck for nutrient entry into the endosperm.
26 127 Subsequently, amino acids are transported into the starchy endosperm where they are
27 128 utilized for protein synthesis (Wang *et al.*, 1995b). The epithelium of the scutellum also
28 129 takes up nutrients from the apoplast to support embryo development and protein
29 130 synthesis. Therefore, the amino acid transporters in these grain cells may play crucial
30 131 roles in regulating nitrogen accumulation in wheat grains.

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34 132 Our previous work has identified three amino acid transporters (TaAAP2, TaAAP13, and
35 133 TaAAP21) highly expressed in different grain cells (Wan *et al.*, 2017). In this study, their
36 134 expression patterns and roles in amino acid transport and nitrogen accumulation in the
37 135 wheat grains were characterized by heterologous expression and transgenesis.

38 39 40 136 **Results**

41 42 137 **The amino acid transporters TaAAP2, TaAAP13, and TaAAP21 are differentially** 43 138 **expressed in wheat grain cells and plant organs**

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46 140 Previously three TaAAP genes (TaAAP2, TaAAP13, and TaAAP21) out of 100
47 141 homoeologous groups of amino acid transporters (283 genes) were shown to be highly
48 142 expressed in the endosperm transfer cells, starchy endosperm cells and aleurone cells
49 143 of wheat grains, respectively (Wan *et al.*, 2017). These genes were therefore selected to
50 144 determine their gene expression patterns, spatial tissue localization and potential
51 145 functions in grain nitrogen metabolism.
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3 147 The expression patterns determined from RNA-seq data of endosperm transfer cells,
4 148 starchy endosperm cells, and aleurone cells at 20 DPA (days post anthesis) (Pfeifer *et*
5 149 *al.*, 2014; Wan *et al.*, 2017) are shown in Figure 1a-c. TaAAP2 (three homoeologs with
6 150 the IWGSC RefSeq v1.1 IDs: TraesCS2A02G348600 (TaAAP2A),
7 151 TraesCS2B02G367000 (TaAAP2B), TraesCS2D02G347000 (TaAAP2D)) was highly
8 152 expressed in endosperm transfer cells at 20 DPA (corresponding to the middle of grain
9 153 filling), but had very low expression in starchy endosperm and aleurone cells at 20 DPA
10 154 (Figure 1a). TaAAP13 (TraesCS4A02G215300 (TaAAP13A), TraesCS4B02G100800
11 155 (TaAAP13B), TraesCS4D02G097400 (TaAAP13D)) was highly expressed in starchy
12 156 endosperm and aleurone cells (Fig. 1b) while TaAAP21 (TraesCS7A02G356639
13 157 (TaAAP21A), TraesCS7B02G271151 (TaAAP21B), TraesCS7D02G366000
14 158 (TaAAP21D)) was most abundant in aleurone cells compared with starchy endosperm
15 159 and transfer cells (Fig. 1c). RNA-seq showed that the three AAP genes are more highly
16 160 expressed in developing grains than vegetative organs prior to anthesis (Choulet *et al.*,
17 161 2014) (Supplementary Figure S1). Gene expression was therefore determined in further
18 162 stages of grain development and in vegetative organs of field-grown wheat at two
19 163 nitrogen levels (with fertilizer applied at 0 kg N/ha, and 200 kg N/ha), using RT-qPCR
20 164 (Figure 1d-f). TaAAP2 and TaAAP13 were highly expressed during the middle stages of
21 165 grain filling (14 and 21 DPA), while the expression of TaAAP21 increased during the late
22 166 stage of grain filling (28 DPA). Nitrogen treatment did not greatly affect the expression
23 167 levels of TaAAP2 and TaAAP13 in grains, but negatively affected their expression in
24 168 vegetative organs at some growth stages. The gene expression patterns determined by
25 169 RT-qPCR generally agreed with the RNA-seq data.

170 171 **Localization of TaAAP2, TaAAP13 and TaAAP21 expression by promoter::GUS** 172 **expression.**

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174 Promoter:: β -glucuronidase (GUS) transgenic lines were produced in order to determine
175 precise gene expression patterns. GUS expression was determined in T1 grains of 10
176 lines and in T2 grains of one (TaAAP2 and TaAAP21) or two lines (TaAAP13). These
177 gave similar results which mostly confirmed the expression patterns shown by RNA-seq
178 and RT-qPCR (Figure 2).

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180 TaAAP2B promoter::GUS expression was observed in transfer cells from 7-28 DPA for
181 glasshouse-grown wheat (Figure 2), with the strongest expression at 21 DPA, indicating
182 that TaAAP2 is transfer cell-specific and may play a role in loading amino acids into the
183 starchy endosperm during grain filling. GUS staining of lines expressing the TaAAP13D
184 promoter::GUS was observed in the lobes of the starchy endosperm during early grain
185 development (7 DPA), but the staining was faint and required overnight incubation (Figure
186 2). However, it was highly expressed in the starchy endosperm from 14 to 28 DPA with
187 two hours staining. A clear gradient in intensity of staining from the sub-aleurone cells to
188 the inner starchy endosperm was observed at 14 DPA with the strongest expression
189 being in the sub-aleurone cells at 21 to 28 DPA (Figure 2, Supplementary Figure 2a-b).

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3 190 The inner starchy endosperm cells did not exhibit any GUS staining even after overnight
4 191 incubation. No expression was observed in the aleurone cells (Figure 2, Supplementary
5 192 Figure S2a), demonstrating that TaAAP13 was specific for the starchy endosperm, and
6 193 suggesting that the high expression level in the aleurone cells shown by RNA-seq
7 194 resulted from contamination during tissue preparation (Pfeifer *et al.*, 2014). Similarly, no
8 195 GUS staining was observed in the embryo of TaAAP13 promoter::GUS transgenic lines.
9 196 The TaAAP13 GUS expression patterns imply that it may function in transporting amino
10 197 acid across the starchy endosperm.
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14 199 TaAAP21 promoter::GUS activity was detected in the epithelium of the scutellum, the
15 200 aleurone, and the transfer cells (Figure 2, Supplementary Figure S2c-f). GUS staining
16 201 was very weak at 14 DPA (Figure 2k), but increased in both, the aleurone and the
17 202 epithelium of the scutellum from 21 to 28 DPA (Figure 2, Supplementary Figure 2c-f),
18 203 which is consistent with the data from RT-qPCR and RNA-seq. However, TaAAP21 was
19 204 much more strongly expressed in the epithelium of the scutellum and root primordia, but
20 205 less in the aleurone at 33 DPA. The expression pattern of TaAAP21 indicates that
21 206 TaAAP21 may transport amino acids into aleurone cells for storage protein synthesis,
22 207 and uptake amino acids into the scutellar epithelium for the embryo development.
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27 209 No GUS staining with the three promoter::GUS constructs was observed in leaf, root or
28 210 stem tissues of plants grown in the glasshouse (well-watered with high nitrogen) over two
29 211 generations. Very weak GUS expression was detected in the tips of the roots in
30 212 germinating seeds after 2-3 days imbibition in water (Supplementary Figure S2g-i).
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33 214 **The amino acid transporters are able to transport broad ranges of amino acids in** 34 215 **yeast mutants.**

35 216 Yeast (*Saccharomyces cerevisiae*) has been used as a heterologous expression system
36 217 to characterize many plant transporters, using mutant strains that lack transporters for
37 218 specific essential components e.g. amino acids. In order to functionally characterize and
38 219 determine the selectivity of TaAAP2, TaAAP13, and TaAAP21, yeast mutants lacking
39 220 transporters for 17 endogenous amino acids were transformed with plasmids containing
40 221 the full length gene coding regions of the three wheat amino acid transporters and growth
41 222 of transformants were tested on media containing different amino acids as sole nitrogen
42 223 sources or as sole source for lysine. This showed that all three wheat transporters can
43 224 transport a broad spectrum of amino acids, some of which are shared (Supplementary
44 225 Table S2, and Supplementary Figure S3). Most of amino acids transported by the three
45 226 amino acid transporters are neutral amino acids (Pro, Gln, Gly, Leu, Ile, Met, Phe, Val,
46 227 Thr, Trp, and Tyr), but acidic (Glu and Asp) or basic (Arg and Lys) amino acids are also
47 228 transported. TaAAP2 can transport a wide range of amino acids, particularly uncharged
48 229 amino acids, but cannot transport Gln, Gly (uncharged), Glu (acidic), Lys or Arg (basic).
49 230 By contrast, TaAAP13 and TaAAP21 can transport Gln, which is the major transported
50 231 amino acid in plants, Glu and other neutral, basic and acidic amino acids.
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233 **Functional analysis of amino acid transporter TaAAP13 by RNAi-suppression**

234 TaAAP13 is highly expressed in the starchy endosperm and hence may provide amino
235 acid substrates for storage protein synthesis in this tissue. Transgenic RNA interference
236 (RNAi) lines were therefore generated to explore its role in more detail. The RNA
237 expression levels in T3 grains of three TaAAP13 RNAi lines (SE-1R, 9R, 10R) at 14 DPA
238 (the stage of peak expression) were reduced 44-70% compared with null lines from the
239 same transformation events (SE-3N, SE-11N) and with a non-transgenic control line (wild
240 type, SE-24WT) (Figure 3a). There are no significant differences between RNAi and null
241 lines in grain numbers, thousand grain weight (TGW), biomass, grain yield per plant and
242 grain areas except the SE-10R grain had a greater area than the SE-11N grain
243 (Supplementary Figure S4).

244
245 To determine whether the accumulation of total nitrogen in the grains was reduced, the
246 nitrogen concentrations in wholegrain and white (starchy endosperm) flours of RNAi lines
247 were compared with those of the null lines. Although the concentrations of nitrogen in the
248 RNAi lines were slightly lower than in the nulls, the differences were not statistically
249 significant (Figure 3b). To determine if the nitrogen gradient across the grain was altered,
250 five pearling fractions (F1, F2, F3, F4, and F5, enriched in the bran, aleurone, sub-
251 aleurone, outer starchy endosperm and inner starchy endosperm respectively) and the
252 remaining core of two RNAi (SE-1R, SE-10R) lines were compared with null and wild type
253 lines. The pearling fractions corresponded to 4, 7, 7, 12 and 10% of the grain weight, and
254 the remaining cores to 60% of the total grain weights of the lines. No significant
255 differences in the concentrations of nitrogen in the fractions were observed between the
256 two RNAi lines (SE-1R and SE-10R), or between the RNAi lines and the null (SE-3N) and
257 non-transgenic control (SE-24WT) lines (Supplementary Figure 5a). However, the total
258 concentration of free amino acids determined by HPLC was higher in RNAi line SE-1R
259 (Supplementary Figure 5b), which had the highest level of RNA suppression (Figure 3a).

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261 Whole grain and pearling samples from RNAi SE-1R and the corresponding null line (SE-
262 3N) were therefore analyzed for polar metabolites (which include 11 amino acids) using
263 ¹H-NMR spectroscopy (Supplementary Table S3a,b). Supervised multivariate analysis
264 (OPLS-DA) clearly separated the wholegrain flours from the SE-1 and SE-3 lines (Figure
265 4a), with the score contribution (Figure 4b) showing that the majority of metabolites were
266 significantly elevated in SE-1R ($P < 0.05$, F-test): glutamine by 87%, asparagine by 57%,
267 glycine by 50%, maltose by 279%, and glucose by 105% (Supplementary Table S3a,b).
268 The compositions of the pearling fractions from the two lines were compared by PCA
269 (Figure 4c). The different fractions are separated in PC1, which accounts for 75% of the
270 total variation, while the two lines are separated in PC2, which accounts for 14% of the
271 total variation. The loading plot for PC1 (Figure 4d) shows that the concentrations of all
272 metabolites decreased from the bran (F1) to the core in both lines (SE-1 and SE-3),
273 except for maltose which increased from F1 to F5 (Supplementary Figure 5c,
274 Supplementary Table S3a, b). The loading plot for PC2 shows that amino acids
275 accumulated to higher concentrations in SE-1R than in SE-3N, with glutamine, aspartate,

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3 276 and glycine being increased by 16%-52%, 29.9-41.8%, 21-62%, respectively, in the
4 277 different fractions. The results therefore demonstrated that the concentrations of most of
5 278 the amino acids transported by TaAAP13 were elevated by the suppression of TaAAP13
6 279 in line SE-1R.

7 280
8 281 SDS-PAGE of total protein extracts showed that the total content of gluten proteins was
9 282 significantly reduced only in one RNAi line (SE-1R) (Supplementary Figure S6a, b), where
10 283 it was associated with a reduced proportion of ω -gliadins and increased proportion of
11 284 other gliadins (Supplementary Figure S6c).

285 286 **Overexpression of TaAAP13 increases grain nitrogen content and grain size**

12 287 To determine whether overexpression of TaAAP13 can increase nitrogen accumulation
13 288 in wheat grain, transgenic plants were generated using TaAAP13 driven by two
14 289 promoters: the starchy-endosperm specific wheat high molecular weight glutenin subunit
15 290 (HMW-GS) 1Dx5 promoter and the constitutive maize ubiquitin promoter. Expression
16 291 under the control of the ubiquitin promoter did not affect grain nitrogen content
17 292 (Supplementary Figure S6a). By contrast, expression under the control of the HMW-GS
18 293 promoter significantly increased grain nitrogen concentration, grain size, thousand grain
19 294 weight, and nitrogen content per grain (Figure 5).

20 295
21 296 With this promoter, the TaAAP13 expression levels in 21DPA caryopses were increased
22 297 9-12 fold in transgenic lines P16-OE and P22-OE (containing 6 copies) and by 30-50 fold
23 298 in lines P23-OE (28 copies), P24-OE, P25-OE and P26-OE (12-16 copies) compared with
24 299 a null line (P15-null) and a non-transgenic line (Cadenza, P29-WT) (Figure 5a). These six
25 300 over-expression lines all had increased concentrations of grain nitrogen compared to the
26 301 null and control lines, with statistically significant increases of 14.4% to 32.4% in P23-OE,
27 302 P24-OE and P26-OE (Figure 5b). The thousand grain weight (TGW), N content per grain
28 303 and grain size were also significantly increased by 19.3-31.7%, 31.2-72.3% and 9.3-
29 304 34.7% ($P < 0.05$, F-test), respectively, in P16-OE, P23-OE, P24-OE, and P26-OE (Figure
30 305 5c, 5d, Supplementary Table S4). However, the grain numbers per plant, grain yields per
31 306 plant and plant biomass were significantly decreased by 20.4 and 71.0%, 9.1 and 62%,
32 307 4.3% and 40.9% ($P < 0.05$, F-test) respectively, in the overexpression lines (P23-OE and
33 308 P24-OE) (Supplementary Figure S7).

34 309
35 310 The grains were longer and wider, but more wrinkled in the overexpression lines with
36 311 higher transgene copy numbers and higher expression of TaAAP13 (Figure 5f). To
37 312 determine if the shriveled grains resulted from reduced accumulation of starch, three
38 313 overexpression lines (P26-OE, P23-OE, and P24-OE) with significantly increased
39 314 nitrogen concentrations in grains were selected and their total starch contents compared
40 315 with P15-null and non-transgenic lines (P29-WT) (Figure 5e). The results showed that
41 316 overexpression of TaAAP13 resulted in lower concentrations of total starch by 6.6-14.3%
42 317 and by 9.8-17.3% compared to the null and non-transgenic lines, respectively, but did not
43 318 affect the starch content per grain (Supplementary Table S5). To determine whether the

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3 319 distribution of protein and starch was altered by overexpression of TaAAP13, thin sections
4 320 of whole mature grains were observed by light microscopy. This showed some protein
5 321 bodies were fused to form a larger matrix in the sub-aleurone cells of the dorsal and lobe
6 322 regions of overexpression line P23-OE with less starch granules compared to P15-null
7 323 line (Figure 6), indicating that overexpression of TAAAP13 also affected the distribution
8 324 of protein and starch in the starchy endosperm. SDS-PAGE showed that overexpression
9 325 of TaAAP13 increased ω -gliadins and other gliadins (Supplementary Figure S8a), with a
10 326 significant increase in the proportion of ω -gliadins in three lines and a significant decrease
11 327 in the proportion of HMW subunits in two of the lines (Supplementary Figure S8b).
12 328

13 329 Determination of the profiles of polar metabolites from wholegrain flours using $^1\text{H-NMR}$
14 330 spectroscopy showed that the concentrations of 13 of the 15 free amino acids that were
15 331 determined, including glutamine, proline and aspartic acid which were most abundant,
16 332 were increased in the overexpression lines (P23-OE and P24-OE), by 1.5 to 2.2 fold
17 333 compared with the null and wild type lines (Supplementary Figure S9). Increases were
18 334 also observed in the most abundant soluble sugars: glucose, fructose and sucrose. The
19 335 overexpression of TaAAP13 therefore supported the results of the yeast
20 336 complementation, suggesting an ability to transport a broad range of amino acids *in*
21 337 *planta*. Partial least squares discriminant analysis (PLS-DA) of 28 metabolites (including
22 338 15 amino acids) showed that the first two X-variables (accounting for 72.8% of the
23 339 explained variation) separated the six overexpression lines from the P15-null line and
24 340 the non-transgenic P29-WT line (Cadenza) (Supplementary Figure S10a). In particular,
25 341 the control lines are positively associated with X-variate 1 and negatively associated with
26 342 X-variate 2. These X-variables are defined by their loadings, shown in Supplementary
27 343 Figure S10b. The results indicate that overexpression of TaAAP13 also changed the
28 344 profiles of metabolites in the grains by accumulation of more free amino acids and soluble
29 345 sugars in transgenic lines compared to the null or wild type lines.
30 346

31 347 Discussion

32 348 Nutrients (amino acids, sucrose, and monosaccharides) are transported from the
33 349 vascular bundle in the crease into transfer cells in the nucellar projection, where they are
34 350 released into the endosperm cavity (Wang *et al.*, 1994b), and subsequently taken up by
35 351 the endosperm transfer cells (as shown schematically in Figure 7). The endosperm
36 352 transfer cells are highly specialized cells with secondary wall ingrowth, which can amplify
37 353 the membrane area up to 20-fold at 25 DPA and consequently enhance the efficiency
38 354 and capacity of transport of solutes (Wang *et al.*, 1994a, Wang, *et al.*, 1995a). In this
39 355 study, TaAAP2 was highly expressed in the endosperm transfer cells (ETC) during grain
40 356 filling (14-28 DPA), which is associated with increased ingrowth of the endosperm transfer
41 357 cell walls (Zheng and Wang, 2011) and increased protein synthesis in the starchy
42 358 endosperm (Shewry *et al.*, 2012). Yeast complementation confirmed that TaAAP2 could
43 359 transport a broad range of neutral amino acids, which was consistent with previous results
44 360 (Fischer *et al.*, 1995, 2002; Okumoto *et al.*, 2002; Rentsch *et al.*, 2007). In addition, most
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3 361 AAPs are located in the plasma membrane (Okumoto *et al.*, 2004) and energized by H⁺
4 362 symporter (De Jong and Borstlap, 2000). Therefore, TaAAP2 expression may be
5 363 coordinated with increased membrane surface area in the endosperm transfer cells to
6 364 regulate the amino acid uptake rate to meet the demand of grain protein synthesis (Figure
7 365 7).
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10 366 The embryo is isolated symplastically from the endosperm, and the epithelium cells of the
11 367 scutellum function as transfer cells (Negbi, 1984) that can transport amino acids from the
12 368 apoplast into the embryo (Figure 7b). The aleurone layer has high concentrations of
13 369 vitamins, minerals, proteins and lipids (Geisler-Lee and Gallie, 2005). The differentiation
14 370 of aleurone cells is initiated at 6-8 DPA and the accumulation of proteins and minerals
15 371 occurs between about 11-27 DPA (Gillies *et al.*, 2012; Xiong *et al.*, 2013). TaAAP21 was
16 372 most strongly expressed in the aleurone and scutellar epithelium between 14-28 DPA,
17 373 and can complement many amino acids in yeast mutants, implying a role in transport of
18 374 amino acids into the embryo and aleurone (Figure 7). This is supported by data on the
19 375 *Arabidopsis* ortholog, AtAAP1, which is expressed in the epidermal transfer cells of the
20 376 embryo and endosperm, thereby facilitating the import of amino acids into the embryo
21 377 (Sanders *et al.*, 2009). However, the functions of TaAAP2 and TaAAP21 in transporting
22 378 amino acids in the wheat grain need to be confirmed by direct functional analysis.
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27 379 Amino acids are transported into the starchy endosperm for storage protein synthesis,
28 380 and endosperm protein content is dependent on amino acid availability. TaAAP13 was
29 381 expressed in the lobes of the starchy endosperm during early endosperm development,
30 382 expressed more strongly in the sub-aleurone and inner starchy endosperm during the
31 383 middle phase of grain development, but restricted to the sub-aleurone cells during late
32 384 grain development. The highest level of expression of TaAAP13 in wheat endosperm was
33 385 at 14 DPA at the start of the grain filling period and about a week before the maximum
34 386 rate of protein accumulation (at about 21 DPA) (Wan *et al.*, 2008, 2013, 2014). ω -gliadins,
35 387 α -gliadins, and low molecular weight subunits of glutenin are mainly deposited in the sub-
36 388 aleurone cells and show a gradient from the outer cell layers to the inner endosperm
37 389 determined by RNA *in-situ* hybridization, promoter::GUS and protein
38 390 immunofluorescence (Van Herpen *et al.*, 2008; Tosi *et al.*, 2011; Wan *et al.*, 2014).
39 391 TaAAP13 showed similar expression patterns and localization to storage proteins.
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44 392 TaAAP13 can transport 15 amino acids by yeast complementation, notably glutamine,
45 393 which comprises 50–60% of the free amino acids in the wheat caryopses at 7 DPA
46 394 (Howarth *et al.*, 2008) and is present in the endosperm cavity (43% of the free amino acid
47 395 pool) at 21 DPA (Fisher and Macnicol, 1986). In addition, glutamine accounts for 30 and
48 396 50% of wheat amino acid residues in the gluten proteins stored in the wheat starchy
49 397 endosperm (Shewry and Halford, 2002). This suggests that glutamine availability is very
50 398 important for gluten protein synthesis.
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53 399 Suppression of TaAAP13 expression did not reduce the total concentration of nitrogen in
54 400 the mature grain or the radial distribution shown by pearling, but did result in increased
55 401 concentrations of free amino acids (especially glutamine, glycine and asparagine) in both
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3 402 wholegrain flour and pearling fractions. A similar effect on total free amino acids was also
4 403 observed in UMAMIT 11, 14, 28 and 29 loss-of-function mutants of Arabidopsis (Müller
5 404 *et al.*, 2015). The concentrations of amino acids were also increased by more in the inner
6 405 starchy endosperm and core fractions produced by pearling.

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9 406 The amino acids taken up by endosperm transfer cells are transported across the starchy
10 407 endosperm tissue to the sub-aleurone cells via a coordination of symplastic and
11 408 apoplastic routes (De Jong and Borstlap, 2000, Wang, *et al.*, 1995a). However, there is
12 409 no direct evidence to prove the cellular pathway of amino acid transport in wheat starchy
13 410 endosperm. Ugalde and Jenner (1990) reported that the amino acid concentrations of
14 411 dorsal endosperm showed a decrease from the endosperm cavity to the midpoint due to
15 412 the influence of high concentrated fluid in endosperm cavity, followed by an increase from
16 413 the midpoint to the periphery. However, in this study, the concentrations of 11 free amino
17 414 acids increased from the endosperm core to the aleurone cells (present in pearling
18 415 fraction F2) in both the RNAi and null lines (Supplementary Table S3a), indicating that
19 416 amino acid transporters are required to transport nitrogen to the outer endosperm against
20 417 gradients in amino acid concentrations. Most known Arabidopsis AAPs are active, proton-
21 418 coupled amino acid symporters (Fischer *et al.*, 1995; Lohaus and Fischer, 2002; Tegeder
22 419 and Ward, 2012). We therefore suggest that suppression of TaAAP13 expression
23 420 reduced the import of the free amino acids (glutamine, glycine, and asparagine) into the
24 421 sub-aleurone cells, which subsequently resulted in greater accumulation of free amino
25 422 acids in the inner endosperm cells in the RNAi line compared to the null line (Figure 7).
26 423 The repression of AtAAP1 function in seed loading Arabidopsis clearly decreases N
27 424 import into the embryo, which in turn leads to an increase in the free amino acid pool in
28 425 the pericarp, endosperm and most probably also in the seed apoplast (Sanders *et al.*,
29 426 2009). However, the concentrations of amino acids were also increased in mature grains
30 427 of lines with overexpression of TaAAP13 under the control of HMW-GS. This suggests
31 428 that amino acid transport capacity was increased by overexpressing TaAAP13, with more
32 429 total nitrogen accumulation in individual grains. Free amino acids were increased both in
33 430 RNAi and overexpression lines of TaAAP13, but the patterns of accumulation are
34 431 different.

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41 432 The overexpression of TaAAP13 not only increased the nitrogen concentration and the
42 433 nitrogen content per grain, but also increased the concentration of gluten proteins,
43 434 particularly ω -gliadins. These results suggest that TaAAP13 may play a role in importing
44 435 amino acids into the endosperm for storage protein synthesis during the middle and late
45 436 grain filling stages. The ectopic expression of HvSUT in wheat endosperm and VfAAP1
46 437 in pea cotyledons resulted in increased accumulation of gliadins and globulins in seeds
47 438 respectively (Rolletschek *et al.*, 2005; Weichert *et al.*, 2010). By contrast, the proportion
48 439 of ω -gliadins was reduced in the TaAAP13 RNAi line. The greater effect of TaAAP13 on
49 440 ω -gliadins is consistent with the ω -gliadins being highly responsive to the nitrogen status
50 441 (Wan *et al.*, 2014).

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3 442 The higher thousand grain weight (TGW) and larger grain size in the OE lines resulted
4 443 mainly from the increased grain protein content. Although the starch concentration was
5 444 decreased, the starch content per individual grain was not reduced. The remobilization
6 445 and translocation of nitrogen to spikes strongly affects grain number (Santiago and
7 446 Tegeder, 2016) and TGW and grain size showed both negative relationship with grain
8 447 number per plant in the OE lines. This suggests that the increased sink capacity of the
9 448 OE grain resulted in the redistribution of the limited nutrient supply into a smaller number
10 449 of larger grains. Previous studies have shown that TGW and grain size were greatly
11 450 increased by ectopic expression of barley sucrose transporter (HvSUT) in wheat grain
12 451 (Weichert *et al.*, 2010) and of VfAAP1 in peas (Rolletschek *et al.*, 2005), with grain
13 452 number per spike being decreased in wheat. Grain size is associated with the numbers
14 453 and size of cells in the grain. Expressing the Arabidopsis phloem-specific sucrose
15 454 transporter (AtSUC2) in rice phloem increased grain size, which resulted mainly from an
16 455 increase in cell size and not cell number for large endosperm (Wang *et al.*, 2015). In
17 456 TaAAP13 OE grains, more large cells filled with protein matrix were observed in the sub-
18 457 aleurone, which may have contributed to the increased grain size. Unexpectedly, the plant
19 458 biomass decreased in the OE lines, and it is possible that the strong sink for nitrogen in
20 459 the endosperm could have affected embryo development by reducing the nutrient supply,
21 460 which consequently had a negative impact on plant biomass. In contrast with the OE
22 461 lines, no effects of TaAAP13 on TGW, total grain nitrogen content or grain size were
23 462 observed in the RNAi lines. This may be because the RNAi suppressed TaAAP13
24 463 expression in whole plants, not only in the grain. However, more work is required to further
25 464 investigate the mechanisms determining increased grain size, nitrogen accumulation,
26 465 and their impact on plant biomass of OE lines in the future.

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34 466 The study therefore suggests that overexpression of TaAAP13 in the starchy endosperm
35 467 increased sink strength and hence grain size and weight by increasing the nitrogen
36 468 uptake capacity of the grain. However, this resulted in reduced grain number due to
37 469 limited availability of assimilate. This suggests that increases in grain nitrogen content
38 470 and grain yield would require simultaneous increases in remobilized nitrogen to spike and
39 471 in importing nitrogen in grain for future wheat breeding

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43 44 473 **Experimental procedures:**

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46 474 **Expression analysis by RNA seq.** Expression data for three amino acid transporters
47 475 were extracted from RNA-seq data (Wan *et al.*, 2017; Pfeifer *et al.*, 2014; Choulet *et al.*,
48 476 2014). Expression units are in FPKM (frequency per kilobase million).

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50 477 **Harvest of materials and RNA extraction.** The wheat cultivar Hereward was grown in
51 478 field trials at Rothamsted Research in 2015, with either 200 kg N/ha fertilizer nitrogen or
52 479 no nitrogen application. For the 200 kg N application, 50, 100, and 50 kg N/ha nitrogen
53 480 (as ammonium nitrate) were applied at tillering, stem extension, and flag leaf emergence
54 481 stages, respectively. Whole caryopses were harvested at 5, 10, 14, 17, 21, and 28 DPA

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3 482 (days post anthesis), and roots and leaves and stems at Zadoks 23 (2–3 tillers stage),
4 483 Zadoks 45 (booting stage), and 14 DPA and stored at -80°C for subsequent RNA
5 484 extraction and real-time PCR.

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8 485 RNA extraction was modified as described by (Chang *et al.*, 1993). Frozen tissues were
9 486 ground in liquid nitrogen and extracted in CTAB buffer (2% (w/v) CTAB, 2% (w/v) PVP
10 487 K30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/l spermidine, 2% (w/v)
11 488 β -mercaptoethanol). The supernatant was extracted twice with chloroform:IAA (24:1) to
12 489 remove proteins. RNA was precipitated by addition of 0.25 volumes of 10 M LiCl and
13 490 incubation on ice overnight. The RNA pellet was dissolved in SSTE buffer (1.0 M NaCl,
14 491 0.5% (w/v) SDS, 10 mM Tris HCl pH 8.0, 1 mM EDTA) to remove polysaccharides and
15 492 extracted once with chloroform:IAA. After ethanol precipitation, total RNA was dissolved
16 493 in DEPC-treated water and stored at -80°C .

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20 494 Total RNA was treated with DNAase to remove genomic DNA contamination and purified
21 495 through RNeasy mini spin columns (Qiagen). Two μg of RNA was used for reverse
22 496 transcription with SuperScriptTM III reverse transcriptase (Invitrogen) to synthesize cDNA
23 497 using anchored oligo (dT) 23 primers (Sigma-Aldrich).

25 498 **Real time PCR.** Real time PCR was performed using an ABI7500 (Applied Biosystems)
26 499 thermocycler. cDNA diluted 1:5 was used for RT-qPCR in a 25 μl reaction with SYBR @
27 500 Green JumpStartTM Taq ReadyMix (Sigma-Aldrich).

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30 501 The Cell division control protein AAA-superfamily ATPases (TraesCS4A02G035500) was
31 502 used as an internal control gene as it showed the most stable expression across different
32 503 wheat tissues and developmental stages (Paolacci *et al.*, 2009). The primers designed
33 504 for RT-qPCR are shown in Supplementary Table S1. For each pair of primers, PCR
34 505 efficiency was calculated in each run from a pool of all available cDNAs by using the
35 506 LinRegPCR software⁷⁹. All time points had three biological replicates. Normalised
36 507 relative quantity (NRQ) was calculated by CT values and primer efficiency (E) of the target
37 508 gene (T) in relation to the internal control gene (N) based on Rieu and Powers (2009) as
38 509 following formula: $\text{NRQ} = 1000 \times (E_{(T)})^{-\text{CT}_{T}} / (E_{(N)})^{-\text{CT}_{N}}$.

41 510 **Plasmid vector construction.** For promoter::GUS constructs, 1.412, 1.582 and 1.178
42 511 kb promoter fragments of TaAAP2B (B-genome), TaAAP13D (D-genome), and
43 512 TaAAP21A (A-genome) were amplified from wheat leaf genomic DNA and cloned into
44 513 pGEM-T Easy vector (Sigma), and then subcloned into vector pRRes104.293 containing
45 514 β -glucuronidase (GUS) with PmeI and NcoI restriction sites. For the RNAi plasmid
46 515 construct of TaAAP13, a 538bp fragment (125 to 662bp from ATG) of the coding region
47 516 was cloned into pGEM-T Easy vector, then subcloned into the RNAi cassette in
48 517 pRRes104RR.132 which contains with two pairs of restriction enzyme sites of BgIII/BsrGI
49 518 and BamHI/BsiWI surrounding the maize ADH2 (alcohol dehydrogenase) intron.
50 519 Expression is driven by the maize ubiquitin promoter plus intron and Nos terminator. For
51 520 overexpression lines of TaAAP13, its full length of coding region was subcloned into
52 521 vector pRRes104RR.161 under the control of the wheat endosperm-specific DX5 HMW-

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3 522 GS promoter or vector pRRes104RR.125 under the control of the constitutive maize
4 523 ubiquitin promoter plus intron and Nos terminator.

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6 524 **GUS assay.** Fresh grains at 7, 14, 21, 28 and 33 DPA were cut transversely or
7 525 longitudinally and incubated in staining buffer (1 mM X-Gluc, 100 mM sodium phosphate
8 526 pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 2% Triton X-
9 527 100) at 37°C for two hours to overnight. Roots, leaves and stems were vacuum-infiltrated
10 528 for 5 minutes before incubation in staining buffer. GUS-stained tissues were visualized
11 529 directly or after de-staining with ethanol and photographed using a Leica MA250 camera.

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14 530 **Wheat Transformation.** The wheat cultivar Cadenza was transformed using particle
15 531 bombardment to deliver plasmid DNAs into immature embryos and transgenic plants
16 532 regenerated via somatic embryogenesis (Sparks and Jones, 2014). Transgenic plants
17 533 were confirmed by PCR using gene-specific primers. Transformed plants were grown to
18 534 maturity in the GM glasshouse.

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21 535 **Homozygous assay and growth of transgenic plants.** DNA was extracted from leaves
22 536 of T1 seedlings and used for determination of homozygosity and transgene copy numbers
23 537 by iDna Genetics Ltd (Norwich Research Park, UK). Wheat plants were grown in a GM
24 538 glasshouse with 16 hours day at 20°C and 8 hours night at 15°C. The plants were watered
25 539 once a day using a flood bench system lined with capillary matting. Four biological
26 540 replicates comprising 4 plants each were grown in a randomized order.

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29 541 **Complementation of yeast amino acid transporter mutants.** Full length coding
30 542 regions of TaAAP2, TaAAP13 and TaAAP21 were PCR amplified from cDNA of 21 DPA
31 543 wheat grains, cloned into pGEM-T Easy vector and confirmed by sequencing. The three
32 544 AAP genes were subcloned into vector PDR196 between a PMA (plasma membrane H⁺-
33 545 ATPase) promoter and ADH terminator, and *Saccharomyces cerevisiae* strains 22Δ8AA,
34 546 22 Δ6AAL, YDR544.137, 30.537a, 21.983c, and 22574d were transformed according to
35 547 Dohmen *et al.*, 1991. The transformants, negative control (empty vector) and positive
36 548 controls (containing Arabidopsis AAPs) were selected on nitrogen-free media containing
37 549 1g/l for proline, aspartate, glutamate and GABA, 1mM for citrulline, glycine, Isoleucine,
38 550 methionine, phenylalanine, and valine, 5mM for leucine, threonine, tryptophan and
39 551 tyrosine, 0.5g/l for arginine, and 0.1mM for lysine with 1g/l Urea. For non-selective
40 552 conditions, media were supplemented with 0.5g/l ammonium sulfate and grown at 30°C
41 553 for 5-7 days (Fischer *et al.*, 2002).

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44 554 **Nitrogen determination.** Total nitrogen was determined using the American Society for
45 555 Testing and Materials (ASTM) standard protocol E1019 using a Leco combustion analysis
46 556 system based on the Dumas method. 150 mg of milled wheat grain and four biological
47 557 replicas were analyzed.

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50 558 **Pearling of grains.** 20g mature seeds were pearled in a Streckel & Schrader (Hamburg,
51 559 Germany) pearling mill (based on Tosi *et al.*, 2011). Five fractions were prepared by
52 560 sequential pearling, corresponding to about 4, 7, 7, 12 and 10 % of the grain weight, and
53 561 the remaining cores (60%) and whole grains were milled in a centrifugal mill (Retsch,

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3 562 ZM200). These pearled fractions are enriched in embryo and pericarp tissue (F1),
4 563 aleurone layer (F2), sub-aleurone layer (F3) and two progressively more central areas of
5 564 the starchy endosperm (F4 and F5), respectively. Three biological replicates were
6 565 performed.

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9 566 **Grain area, length, width, moisture and biomass measurement.** Seed parameters of
10 567 area (size), length and width were measured using MARVIN- Digital Seed Analyzer SN
11 568 176 (Marvitech - Germany). Four biological replicates from 16 plants (200-400 grains from
12 569 each biological replicate) were analyzed. Seed moisture was detected using Bruker
13 570 Minispec mq20 TD-NMR Contrast Agent analyzer (Germany) with bespoke robot and
14 571 recording software (ROHASYS, Dutch Robotics) by loading 7-10g seeds. Thousand grain
15 572 weight (TGW) was converted into 15% seed moisture grain weight. Biomass (above
16 573 ground stems and leaves) were oven dried at 80°C overnight.

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21 575 **SDS-PAGE.** 10mg flour was suspended in 300µl total protein extraction buffer [50 mM
22 576 Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 2 % (w/v) dithiothreitol (DTT) and
23 577 0.1 % (w/v) bromophenol blue] (Wan *et al.*, 2014). The extracts were heated for 3 minutes
24 578 at 95°C, and centrifuged for 15 minutes. 3 µl of the supernatants were separated on pre-
25 579 cast 4–12 % Bis-Tris Nu-PAGE gels (Invitrogen). The four biological extracts, and two
26 580 technical gel running were repeated. The gels were stained with Coomassie BBR250 in
27 581 10 % (w/v) trichloroacetic acid (TCA), 40 % (v/v) methanol, and de-stained in 10 % (w/v)
28 582 TCA. The gels were scanned with a HPG4010 scanner, and the images from grey tif files
29 583 were processed with Phoretix 1D advanced software (Nonlinear Dynamics, Durham, NC,
30 584 USA). Each group protein percentage is expressed as a % of total gluten proteins.

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34 585 **¹H-Nuclear magnetic resonance (NMR) spectroscopy.** ¹H-NMR sample preparation
35 586 was carried out according to (Baker *et al.*, 2006). Wholegrain samples (30 mg) (three
36 587 technical replicates each of three biological replicates) were extracted in 80:20
37 588 D2O:CD3OD containing 0.05% d4-trimethylsilylpropionate (TSP; 1mL) as an internal
38 589 standard at 50°C for 10 min. After centrifugation (5 min at 13 000 rpm), the supernatant
39 590 was removed and heated to 90°C for 2 min to halt enzyme activity. After cooling and
40 591 further centrifugation, the supernatant (650 µL) was transferred to a 5 mm NMR tube for
41 592 analysis. The data collection and analysis were described as in Shewry *et al.* (2017).

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45 593 **High performance liquid chromatography (HPLC).** Free amino acids were extracted
46 594 according to Curtis *et al.* (2018). 30 mg samples (four biological replicates) were
47 595 suspended in 500 µL of 0.01 N HCl by shaking for 30 min at room temperature. After
48 596 centrifugation at 10000 rpm for 15 minutes, the supernatants were filtered through a 0.45
49 597 µm poly (ether sulfone) filter before analysis. Amino acids were separated using a Waters
50 598 Alliance 2795 HPLC system (Waters Corp., Milford, USA) coupled with a Waters 474
51 599 scanning fluorescence detector. The detailed method is described in Shi *et al.* (2019).

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54 600 **Wheat grain fixation, embedding and light microscopy.** The middle parts of 5 mature
55 601 grains were cut into 2 mm transverse sections and immediately fixed in 4 % (w/v)

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3 602 paraformaldehyde in 0.1 M Sorenson's phosphate buffer ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot$
4 603 $12\text{H}_2\text{O}$, pH 7.0) with 2.5 % (w/v) glutaraldehyde overnight. After dehydration in increasing
5 604 concentrations of ethanol, the sections were embedded in LR White Resin for two weeks
6 605 at room temperature and polymerized at 55°C. A Reichert-Jung Ultracut ultramicrotome
7 606 was used to section the resin-embedded grains at 0.5 μm or 1 μm thickness for protein
8 607 staining. Protein bodies were stained with 1 % (w/v) Naphthol Blue Black in 7 % (w/v)
9 608 acetic acid. The slides were visualized under a Zeiss Axiophot microscope and images
10 609 were acquired with a RetigaExi CCD digital camera (Qimaging, Surrey, BC, Canada)
11 610 under bright field optics and MetaMorph software version 7.5.5 9 (Molecular Devices,
12 611 Sunnyvale, CA, USA).

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16 612 **Total starch assay.** The total starch contents of samples containing resistant starch
17 613 were determined using the Megazyme total starch kit (amyloglucosidase/amylase method,
18 614 Megazyme, Bray, Ireland). Wholegrain flour (100mg) with four biological replicates and
19 615 two technical extracts was extracted with dimethyl sulphoxide, and digested with a
20 616 thermostable alpha-amylase at 100°C for 15 minutes. Amyloglucosidase was added to
21 617 release glucose at 50°C for 30 minutes. The final glucose content was calculated after
22 618 incubation with glucose oxidase/peroxidase and absorbance measurement at 510 nm
23 619 against blank and glucose standards

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27 620 **Statistical analysis.** Data were analyzed using ANOVA accounting for the randomized
28 621 block design. Where necessary, data were log or square root transformed to satisfy
29 622 homogeneity of variance. The least significant difference (LSD) values presented are the
30 623 LSD associated with comparisons. Analyses were done using the GenStat (19th edition,
31 624 VSN International Ltd., Hemel Hempstead, U.K.). Principal component analysis (PCA)
32 625 and partial least squares discriminant analysis (PLS-DA) were applied using the
33 626 correlation matrix between variables. Each input variable was transformed according to
34 627 the univariate analysis, adjusted for any block effects and averaged over technical
35 628 replicates. PCA was performed in Genstat, 19th edition. The software Simca-P v. 16
36 629 (Sartorius Stedim Data Analytics AB) was used for OPLS-DA. The analysis was carried
37 630 out using quantified 1H-NMR data scaled to unit variance.

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636 Rothamsted Bioimaging staff for their support.

637 **Conflict of interests**

638 The authors declare that they have no competing interests.

639 **Author contributions**

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3 640 YFW, PRS, MJH conceived the conception and wrote the manuscript, YFW and PB
4 641 contributed all the constructs, GUS expression, starch assay, YW and ZQS contributed
5 642 to microscopy and protein work, DR performed yeast complementation, JW performed
6 643 ¹H-NMR, CAS and AKH provided transgenic service and plasmid vector cassette, KH
7 644 and SP analyzed the data. All the authors revised the manuscript for submission.

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10 645 **Supporting information:**

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12 646 Supplementary Figure S1. The expression patterns of TaAAP2, TaAAP13, and TaAAP21
13 647 in wheat organs by RNA-seq.

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15 648 Supplementary Figure S2. The localization of TaAAP2, TaAAP13, and TaAAP21
16 649 expression by promoter::GUS analysis.

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18 650 Supplementary Figure S3. Complementation of yeast mutants by TaAAP2, TaAAP13,
19 651 and TaAAP21.

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21 652 Supplementary Figure 4: The agronomic traits of the RNAi lines (SE-1R, SE-9R, SE-
22 653 10R), Nulls (SE-3N, SE-11N), and non-transgenic line (SE-24WT).

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24 654 Supplementary Figure S5. The nitrogen concentration and free amino acids in RNAi lines
25 655 of wholegrain flour and pearling fractions.

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28 656 Supplementary Figure S6. The gluten protein and composition of wholegrain flour by
29 657 SDS-PAGE in RNAi lines (SE-1R, 9R, 10R), nulls (SE-3N, 11N) and non-transgenic line
30 658 (SE-24WT, Cadenza).

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32 659 Supplementary Figure S7. phenotypes of overexpression of TaAAP13 lines.

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34 660 Supplementary Figure S8. SDS-PAGE and protein composition of overexpression lines.

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36 661 Supplementary Figure S9: Metabolites of wholegrain flour from overexpression lines were
37 662 determined by ¹H-NMR.

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39 663 Supplementary Figure S10. A Partial least squares discriminant analysis (PLS-DA) of
40 664 metabolites from overexpression lines by ¹H-NMR.

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42 665 Supplementary TableS1. primers for RT-qPCR and plasmid vector constructs.

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44 666 Supplementary Table S2. The amino acids of TaAAP2, TaAAP13, and TaAAP21
45 667 complementation in yeast mutants.

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47 668 Supplementary Table S3. The metabolite changes in RNAi line (SE-1) and Null (SE-3)
48 669 whole grain flour and their pearling fractions by ¹H-NMR.

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50 670 Supplementary Table S4. The grain size of pHMW-TaAAP13 overexpression lines.

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52 671 Supplementary Table S5: The starch content (mg) /per grain in overexpression lines.

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Figure legends:

867 Figure 1: The expression patterns of TaAAP2, TaAAP13, and TaAAP21.

869 (a-c). Expression of TaAAP2 (a), TaAAP13 (b), and TaAAP21 (c) in grain cells:
870 endosperm transfer cells (ETC), starchy endosperm (SE), aleurone cells (AL) of Chinese
871 Spring at 20DPA (days post anthesis) (Pfeifer et al 2014; Wan et al, 2017) by RNA-seq
872 data. (d-f). Expression of TaAAP2 (d), TaAAP13 (e), and TaAAP21 (f) in different organs
873 of wheat cultivar Hereward from Whole caryopses at 5, 10, 14, 17, 21, and 28 DPA (days
874 post anthesis), roots, leaves and stems at Zadoks 23 (2–3 tillers stage) and Zadoks 45
875 (booting stage) at two nitrogen levels 0kg N/ha and 200kg N/ha by RT-qPCR. Error bars
876 represent standard errors (SE).

877 Figure 2. Localization of TaAAP2B, TaAAP13D, and TaAAP21A by promoter::GUS
878 expression.

879 The GUS expression patterns driven by promoters of TaAAP2B, TaAAP13D, and
880 TaAAP21A in T2 developing grains at 7, 14, 21, 28 and 33DPA (days post anthesis). The
881 GUS staining was visualised directly for TaAAP2B and TaAAP13D or after serial ethanol
882 de-staining for TaAAP21A under stereomicroscope Leica MA250. Scale bars represent
883 500 μm except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 μm . ETC:
884 endosperm transfer cells, EM: embryo, AL: aleurone cells, ES: epithelium of scutellum,
885 SE: starchy endosperm, RP: root primordia.

886 Figure 3. TaAAP13 expression and nitrogen concentrations in RNAi lines (SE-1R, SE-
887 9R, SE-10R), null lines (SE-3N, SE-11N), and non-transgenic line SE-24WT (Cadenza,
888 wild type).

889 (a). TaAAP13 gene expression at 14DPA of wheat developing grain. Asterisks (***)
890 indicated the significant differences between RNAi lines and nulls or wild type detected
891 by ANOVA ($P < 0.001$, F-test). Error bars represent standard errors. (b). Nitrogen
892 concentrations of wholegrain flour and white flour. The differences between RNAi lines
893 and nulls were not statistically significant ($P < 0.05$, F-test).

894 Figure 4. Metabolite data analysis of RNAi and null lines by $^1\text{H-NMR}$.

895 (a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null
896 SE-3N by $^1\text{H-NMR}$. (b). Contribution plot comparing significant component changes
897 from two lines SE-1R and SE-3N; the red colour represents more elevated in SE-3N on
898 the top, and in SE-1R at the bottom. The amino acids (glutamine and glycine) were most
899 elevated in SE-1R, and are indicated by blue arrows. (c). PCA scores plot of pearling
900 fractions: F1, F2, F3, F4, F5, core and wholegrain flour of SE-1R and SE-3N. (d). the
901 loading plot of PCA for pearling fractions. The coloured circles were drawn by separation
902 of different lines in (a) and different fractions in (c), rather than statistically significant. F1,
903 F2, F3, F4, F5 and core are mainly enriched in bran (F1), aleurone cell (F2), sub-aleurone

904 cells (F3), two progressive inner endosperm fractions (F4, F5) and core. (d). Loading plot
905 of (c).

906 Figure 5. The gene expression and phenotype of overexpression TaAAP13 under the
907 control of endosperm-specific promoter HMW-GS Dx5.

908 (a). Gene expression of TaAAP13 at 21DPA in developing grains of overexpression lines:
909 P22-OE and P16-OE (6 copies), P25-OE (16 copies), P26-OE (14copies), P23-OE (26
910 copies), P24 (12 copies), null (P15-null), and non transgenic (P29-WT, Cadenza). (b). N
911 concentration % of wholegrain flour. (c). Thousand grain weight (TGW) at 15% moisture
912 content (g). (d). Nitrogen content (mg) per grain. (e). Total starch concentration in
913 wholegrain flour. (f). Grain morphology. Scale bar represents 1 cm in (f). Significant
914 differences were detected using ANOVA ($P < 0.05$, F-test) and indicated with asterisk
915 (*), and ($P < 0.001$, F-test) with asterisk (**).

916 Figure 6. The protein distribution of mature grain resin sections of TaAAP13
917 overexpression line.

918 (a). Dorsal of P23-OE. (b). Dorsal of P15-null. (c). Lobe of P23-OE. (d). Lobe of P15-null.
919 (e). Whole grain section of P23-OE. (f). Whole grain section of P15-NULL. Resin sections
920 with 1 μm thickness in (a)-(d) and 0.5 μm thickness in (e) and (f) were stained with 1 %
921 (w/v) Naphthol Blue Black in 7 % (w/v) acetic acid. Scale bars represent 50 μm in (a)-(d)
922 and 1mm in (e) and (f). Red arrows Indicated large protein body matrix (PB). AL: aleurone
923 cells. SA: sub-aleurone cells. SG: starch granules.

924 Figure 7. Schematic view of three TaAAPs nitrogen transport. (a). Wheat grain transverse
925 section. (b) wheat grain longitudinal section. VB: vascular bundle. EC: endosperm cavity
926 for amino acids delivered to endosperm from vascular bundle. NPTC: nucellar projection
927 transfer cells. **ETC: endosperm transfer cells**. TaAAP2 expression localization for uptake
928 of amino acids from endosperm cavity. SE: starch endosperm. SA: sub-aleurone cells for
929 proteins (mainly ω and α -gliadins, LMW-glutenin subunits), and TaAAP13 expression
930 localization. **AL: aleurone cells for TaAAP21 expression localization**. **ES: epithelium of**
931 **scutellum for TaAAP21 expression localization**. SC: scutellum. EM: embryo. **↑ : TaAAP2.**
932 **⬆ : TaAAP13.** **▲ : TaAAP21.** **● : protein bodies**

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11 | 2 **Wheat amino acid transporters highly expressed in grain cells regulate grain size**
12 | 3 **and grain nitrogen accumulation**

13 | 4 Yongfang Wan¹, Yan Wang^{1,2}, Zhiqiang Shi^{1,3}, Doris Rentsch⁴, Jane L Ward¹, Kirsty
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Abstract: Amino acids are delivered into developing wheat grains to support the accumulation of storage proteins in the starchy endosperm, and transporters play important roles in regulating this process. RNA-seq, RT-qPCR, and promoter-GUS assays showed that three amino acid transporters (TaAAP2, TaAAP13, and TaAAP21) are differentially expressed in the endosperm transfer cells, starchy endosperm cells, aleurone cells and the embryo of the developing grain, respectively. Yeast complementation revealed that all three transporters can transport a broad spectrum of amino acids. RNAi-mediated suppression of TaAAP13 expression in the starchy endosperm did not reduce the total nitrogen content of the whole grain, but significantly altered the composition and distribution of metabolites in the starchy endosperm, with increasing concentrations of some amino acids (especially glutamine and glycine) from the outer to inner starchy endosperm cells. Overexpression of TaAAP13 under the endosperm-specific HMW-GS promoter significantly increased grain size, grain nitrogen concentration, and thousand grain weight, indicating that the sink strength for nitrogen improvement was increased by manipulation of amino acid transporters. However, the total grain number was reduced, suggesting that source nitrogen remobilized from leaves is a limiting factor for productivity. Therefore, simultaneously increasing loading of amino acids into the phloem and delivery to the spike would be required to increase protein content while maintaining grain yield.

Keywords: amino acid transporter, wheat grain, overexpression, RNAi, grain nitrogen, grain metabolites, grain size.

Abbreviation: alcohol dehydrogenase (ADH), aleurone cell (AL), amino acid/auxin permease subfamily (AAP), amino acid permease (AAP), amino acid polyamine-choline transporters subfamily (APC), amino acid transporter (AAT), analysis of variance (ANOVA), cetyl trimethylammonium bromide (CTAB), complementary DNA (cDNA), days post anthesis (DPA), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), drug/metabolite transporter (DMT), ethylenediaminetetraacetic acid (EDTA), endosperm transfer cell (ETC), frequency per kilobase million (FPKM), genetically modified (GM), β -glucuronidase (GUS), grain protein content (GPC), high molecular weight glutenin subunits (HMW-GS), high performance liquid chromatography (HPLC), ^1H -Nuclear magnetic resonance (NMR), Isoamyl alcohol (IAA), least significant difference (LSD), Nucellar projection transfer cell (NPTC), orthogonal projections to latent structures discriminant analysis (OPLS-DA), partial least squares discriminant analysis (PLS-DA), plasma membrane H^+ - ATPase (PMA), principal component analysis (PCA), protein body (PB), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), RNA interference (RNAi), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), starchy endosperm (SE), starch granule (SG), thousand grain weight (TGW), trichloroacetic acid (TCA), usually multiple amino acids move in and out transporter (UMAMIT).

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78 Introduction

79 ~~Wheat contributes about 10% of the dietary intake of protein in the UK,~~ and grain protein
 80 content (GPC) is a key determinant of breadmaking quality. Farmers routinely increase
 81 the grain protein content by ~~applying more~~ inorganic nitrogen fertilizers, which increases
 82 the cost of production and may have an adverse environmental footprint. Therefore,
 83 ~~improved~~ nitrogen use efficiency is a long-term strategy for sustainable improvement ~~of~~
 84 wheat productivity and grain protein content.

85 ~~Amino acids are the major transported form of reduced nitrogen in the plant (Palmer et~~
 86 ~~al., 2014; Riens et al., 1991).~~ ~~The transport of amino acids across membranes and~~
 87 ~~translocation from source to sink is mediated by membrane transport proteins: amino acid~~
 88 ~~transporters (AATs). These are classified into two major subfamilies: the Amino~~
 89 ~~Acid/Auxin Permease family (AAP) and the Amino acid-Polyamine-Choline transporters~~
 90 ~~family (APC) (Lee and Tegeder, 2004; Rentsch et al., 2007). In addition, a new group,~~
 91 ~~the Usually Multiple Amino Acids Move In and Out Transporter (UMAMIT) family, has~~
 92 ~~been identified (Denancé et al., 2010), which is part of the Drug/Metabolite Transporter~~
 93 ~~(DMT) family (Jack et al., 2001). More than 110 AAT genes have been identified in~~
 94 ~~Arabidopsis and genome-wide surveys of amino acid transporters have been reported for~~
 95 ~~many plant species including rice (Zhao et al., 2012), poplar (Wu et al., 2015), potato (Ma~~
 96 ~~et al., 2016) and wheat (Wan et al., 2017).~~

97 Only a few AAT genes have been functionally characterized, mainly in Arabidopsis.
 98 Nevertheless, studies indicate that they play important roles in amino acid uptake into
 99 roots, phloem loading, long-distance transport and loading into the seed (Dinkeloo *et al.*,
 100 2017; Tegeder, 2012). Crucial functions for ~~the~~ import of amino acids into sink tissues
 101 (seed, fruit, and tuber) have been described for different amino acid transporters. The
 102 AtAAP1 transporter is highly expressed in the embryo epithelium (transfer cells) of
 103 Arabidopsis and involved in uptake and transport of amino acids from the endosperm into
 104 the embryo, with *ataap1* mutants having lower seed protein content and seed weight
 105 (Sanders *et al.*, 2009). RNAi-mediated down-regulation of the StAAP1 transporter can
 106 reduce the levels of free amino acids by 50% in potato tubers (Koch *et al.*, 2003). The
 107 tonoplast-localized UMAMIT24 of Arabidopsis is able to transport the amino acids
 108 temporarily stored in the vacuoles of chalaza cells before being delivered to filial tissue,
 109 while the plasma membrane-localized UMAMIT 25 is expressed in the endosperm and
 110 pericarp and could mediate amino acid export from endosperm (Besnard *et al.*, 2018).
 111 The UMAMIT18 transporter is present in vascular tissues and developing seeds,
 112 supporting accumulation ~~nitrogen~~ in developing siliques (Ladwig *et al.*, 2012). UMAMIT11
 113 and 14 AATs are expressed in the chalaza (unloading domain) of developing seeds and
 114 ~~UMAMIT28 and 29 transporters export amino acids from the endosperm and pericarp,~~
 115 respectively. Single loss-of-function mutants of these four transporters resulted in the
 116 accumulation of high levels of free amino acids in the seeds and greatly reduced seed
 117 size (Müller *et al.*, 2015). By contrast, overexpression of VfAAP1 (which is highly
 118 expressed in embryonic storage parenchyma cells at early maturation) in both pea and

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Deleted: Wheat grains comprise three distinct parts, the embryo, pericarp, and endosperm, which account for about 2.5%, 14%, and 83% of the grain weight, respectively (Pomeranz, 1988). The endosperm consists of three cell types, transfer cells (TC), aleurone cells (AL) and starchy endosperm cells (SE). The starchy endosperm is the main storage tissue and is consumed as white flour after milling to separate the other tissues. ¶

The remobilization of nitrogen in source tissues and its subsequent use in sink tissues are important for producing high grain protein content in wheat (Barneix, 2007). Amino acids are the major transport form of reduced nitrogen in the phloem (Palmer *et al.*, 2014; Riens *et al.*, 1991) and are delivered to the endosperm cavity (Fisher and Macnicol, 1986), taken up by TCs and then transported into the starchy endosperm for storage protein synthesis. ¶

Moved up [1]: Amino acids are the major transport form of reduced nitrogen in the phloem (Palmer *et al.*, 2014; Riens *et al.*, 1991)

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10 154 *Vicia narbonensis* (Narbon bean) seeds resulted in increased seed protein content (by
11 155 10-25%) and seed size (by 20-30%), by increasing import of amino acids into the embryo
12 156 (Rolletschek *et al.*, 2005). Similarly, the simultaneous overexpression of PsAAP1 in the
13 157 phloem and embryos of pea plants increased seed yield and seed storage protein levels
14 158 when the plants were grown with highly abundant N, due to increased source-to-sink
15 159 allocation of amino acids (Zhang *et al.*, 2015) and increased nitrogen use efficiency
16 160 (Perchlik and Tegeder, 2017). The OsAAP6 transporter, which is highly expressed in the
17 161 endosperm of rice, functions as a positive regulator of GPC and overexpression in rice
18 162 greatly increased GPC in rice grain when overexpressed (Peng *et al.*, 2014). Tomato
19 163 SICAT9 is a tonoplast Glu/Asp/GABA transporter, and overexpression of SICAT9 greatly
20 164 affects the flavor profile of the tomato fruit by increasing the accumulation of these amino
21 165 acids during fruit development (Snowden *et al.*, 2015).

22 166 Wheat grains comprise three distinct parts, the embryo, the outer layers (nucellar
23 167 epidermis, testa and pericarp), and the endosperm, which account for about 3%, 7%, and
24 168 90% of the grain weight, respectively (Barron *et al.*, 2007). The endosperm consists of
25 169 three cell types, endosperm transfer cells (ETC), aleurone cells (AL) and starchy
26 170 endosperm cells (SE). Amino acids are unloaded from the phloem via the vascular bundle
27 171 into the endosperm cavity of the grain (Wang *et al.*, 1994b), where they are actively taken
28 172 up by transfer cells; this is the first bottleneck for nutrient entry into the endosperm.
29 173 Subsequently, amino acids are transported into the starchy endosperm where they are
30 174 utilized for protein synthesis (Wang *et al.*, 1995b). The epithelium of the scutellum also
31 175 takes up nutrients from the apoplast to support embryo development and protein
32 176 synthesis. Therefore, the amino acid transporters in these grain cells may play crucial
33 177 roles in regulating nitrogen accumulation in wheat grains.

34 178 Our previous work has identified three amino acid transporters (TaAAP2, TaAAP13, and
35 179 TaAAP21) highly expressed in different grain cells (Wan *et al.*, 2017). In this study, their
36 180 expression patterns and roles in amino acid transport and nitrogen accumulation in the
37 181 wheat grains were characterized by heterologous expression and transgenesis.

38 182 Results

39 183 The amino acid transporters TaAAP2, TaAAP13, and TaAAP21 are differentially 40 184 expressed in wheat grain cells and plant organs

41 185
42 186 Previously, three TaAAP genes (TaAAP2, TaAAP13, and TaAAP21) out of 100
43 187 homoeologous groups of amino acid transporters (283 genes) were shown to be highly
44 188 expressed in the endosperm transfer cells, starchy endosperm cells and aleurone cells
45 189 of wheat grains, respectively (Wan *et al.*, 2017). These genes were therefore selected to
46 190 determine their gene expression patterns, spatial tissue localization and potential
47 191 functions in grain nitrogen metabolism.
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Genes encoding a total of 283 amino acid transporters (AATs) representing 100 homoeologous groups

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Deleted: TaAAP6 is the wheat ortholog of OsAAP6, with the expression level of the most highly expressed homoeoallele (TaAAP6-3B-l) being positively and significantly correlated with GPC (Jin *et al.*, 2018). However, none of the amino acid transporters of wheat have been functionally characterized.

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The expression patterns determined from RNA-seq data of **endosperm** transfer cells, starchy endosperm cells, and aleurone cells at 20 DPA (days post anthesis) (Pfeifer *et al.*, 2014; Wan *et al.*, 2017) are shown in Figure 1a-c. TaAAP2 (three homoeologs with the IWGSC RefSeq v1.1 IDs: TraesCS2A02G348600 (TaAAP2A), TraesCS2B02G367000 (TaAAP2B), TraesCS2D02G347000 (TaAAP2D)) was highly expressed in **endosperm** transfer cells at 20 DPA (corresponding to the middle of grain filling), but had very low expression in starchy endosperm and aleurone cells at 20 DPA (Figure 1a). TaAAP13 (TraesCS4A02G215300 (TaAAP13A), TraesCS4B02G100800 (TaAAP13B), TraesCS4D02G097400 (TaAAP13D)) was highly expressed in starchy endosperm and aleurone cells (Fig. 1b) while TaAAP21 (TraesCS7A02G356639 (TaAAP21A), TraesCS7B02G271151 (TaAAP21B), TraesCS7D02G366000 (TaAAP21D)) was most abundant in aleurone cells compared with starchy endosperm and transfer cells (Fig. 1c). RNA-seq showed that the three AAP genes are more highly expressed in developing grains than vegetative organs prior to anthesis (Choulet *et al.*, 2014) (Supplementary Figure S1). Gene expression was therefore determined in further stages of grain development and in vegetative organs of **field-grown wheat**, at two nitrogen levels (**with fertilizer applied at 0 kg N/ha**, and **200 kg N/ha**), using RT-qPCR (Figure 1d-f). TaAAP2 and TaAAP13 were highly expressed during the middle stages of grain filling (14 and 21 DPA), while the expression of TaAAP21 increased during the late stage of grain filling (28 DPA). Nitrogen treatment did not greatly affect the expression levels of TaAAP2 and TaAAP13 in grains, but negatively affected their expression in vegetative organs at some growth stages. The gene expression patterns determined by RT-qPCR generally agreed with the RNA-seq data.

Localization of TaAAP2, TaAAP13 and TaAAP21 expression by promoter::GUS expression.

Promoter:: β -glucuronidase (GUS) transgenic lines were produced in order to determine precise gene expression patterns. GUS expression was determined in T1 grains of 10 lines and in T2 grains of one (TaAAP2 and TaAAP21) or two lines (TaAAP13). These gave similar results which **mostly** confirmed the expression patterns shown by RNA-seq and RT-qPCR (Figure 2).

TaAAP2B promoter::GUS expression was observed in transfer cells from 7-28 DPA **for glasshouse-grown wheat** (Figure 2), with the strongest expression at 21 DPA, indicating that TaAAP2 is transfer cell-specific and may play a role in loading amino acids into the starchy endosperm during grain filling. GUS staining of lines expressing the TaAAP13D promoter::GUS was observed in the lobes of the starchy endosperm during early grain development (7 DPA), but the staining was faint and required overnight incubation (Figure 2). However, it was highly expressed in the starchy endosperm from 14 to 28 DPA **with two hours staining**. A clear gradient in intensity of staining from the sub-aleurone cells to the inner starchy endosperm was observed at 14 DPA with the strongest expression being in the sub-aleurone cells at 21 to 28 DPA (Figure 2, Supplementary Figure 2a-b).

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261 The inner **starchy** endosperm cells did not exhibit any GUS staining even after overnight
 262 incubation. No expression was observed in the aleurone cells (Figure 2, Supplementary
 263 Figure S2a), demonstrating that TaAAP13 was specific for the starchy endosperm, and
 264 suggesting that the high expression level in the aleurone cells shown by RNA-seq
 265 resulted from contamination during tissue preparation (Pfeifer *et al.*, 2014). Similarly, no
 266 GUS staining was observed in the embryo of TaAAP13 promoter::GUS transgenic lines.
 267 The TaAAP13 GUS expression patterns imply that it may function in transporting amino
 268 acid across the starchy endosperm.

270 TaAAP21 promoter::GUS activity was detected in the epithelium of **the** scutellum, **the**
 271 aleurone, and **the** transfer cells (Figure 2, Supplementary Figure S2c-f). GUS staining
 272 was very weak at 14 DPA (Figure 2k), but increased in both, the aleurone and the
 273 epithelium of the scutellum from 21 to 28 DPA (Figure 2, Supplementary Figure 2c-f),
 274 which is consistent with the data from RT-qPCR and RNA-seq. However, TaAAP21 was
 275 much more strongly expressed in the epithelium of the scutellum and root primordia, but
 276 less in the aleurone at 33 DPA. The expression pattern of TaAAP21 indicates that
 277 TaAAP21 may transport amino acids into aleurone cells for **storage protein synthesis**,
 278 and uptake amino acids into the scutellar epithelium for the embryo development.

280 No GUS staining with the three promoter::GUS constructs was observed in leaf, root or
 281 stem tissues of plants grown in the glasshouse (well-watered with high nitrogen) over two
 282 generations. Very weak GUS expression was detected in the tips of the roots in
 283 germinating seeds after 2-3 days imbibition in water (Supplementary Figure S2g-i).

285 The amino acid transporters are able to transport broad ranges of amino acids in 286 yeast mutants.

287 Yeast (*Saccharomyces cerevisiae*) has been used as a heterologous expression system
 288 to characterize many plant transporters, using mutant strains that lack transporters for
 289 specific essential components e.g. amino acids. In order to functionally characterize and
 290 determine the selectivity of TaAAP2, TaAAP13, and TaAAP21, yeast mutants lacking
 291 transporters for 17 endogenous amino acids were transformed with plasmids containing
 292 the full length gene coding regions of the three wheat amino acid transporters and growth
 293 of transformants were tested on media containing different amino acids as sole nitrogen
 294 sources or as sole source for lysine. This showed that all three wheat transporters can
 295 transport a broad spectrum of amino acids, some of which are shared (Supplementary
 296 Table S2, and Supplementary Figure S3). Most of amino acids transported by the three
 297 amino acid transporters are neutral amino acids (Pro, Gln, Gly, Leu, Ile, Met, Phe, Val,
 298 Thr, Trp, and Tyr), **but** acidic (Glu and Asp) **or** basic (Arg and Lys) **amino acids are also**
 299 **transported**. TaAAP2 can transport a wide range of amino acids, particularly uncharged
 300 amino acids, but cannot transport **Gln**, **Gly** (uncharged), **Glu** (acidic), **Lys** or **Arg** (basic).
 301 By contrast, TaAAP13 and TaAAP21 can transport **Gln**, which is the major transported
 302 amino acid in plants, **Glu** and other neutral, basic and acidic amino acids.

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Deleted: which are highly abundant in the developing wheat grain (Howarth *et al.*, 2008) and the endosperm cavity (Fisher and Macnicol, 1986).

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Functional analysis of amino acid transporter TaAAP13 by RNAi-suppression

TaAAP13 is highly expressed in the starchy endosperm and hence may provide amino acid substrates for storage protein synthesis in this tissue. Transgenic RNA interference (RNAi) lines were therefore generated to explore its role in more detail. The RNA expression levels in T3 grains of three TaAAP13 RNAi lines (SE-1R, 9R, 10R) at 14 DPA (the stage of peak expression) were reduced 44-70% compared with null lines from the same transformation events (SE-3N, SE-11N) and with a non-transgenic control line (wild type, SE-24WT) (Figure 3a). There are no significant differences between RNAi and null lines in grain numbers, thousand grain weight (TGW), biomass, grain yield per plant and grain areas except the SE-10R grain had a greater area than the SE-11N grain (Supplementary Figure S4).

To determine whether the accumulation of total nitrogen in the grains was reduced, the nitrogen concentrations in wholegrain and white (starchy endosperm) flours of RNAi lines were compared with those of the null lines. Although the concentrations of nitrogen in the RNAi lines were slightly lower than in the nulls, the differences were not statistically significant (Figure 3b). To determine if the nitrogen gradient across the grain was altered, five pearling fractions (F1, F2, F3, F4, and F5, enriched in the bran, aleurone, sub-aleurone, outer starchy endosperm and inner starchy endosperm respectively) and the remaining core of two RNAi (SE-1R, SE-10R) lines were compared with null and wild type lines. The pearling fractions corresponded to 4, 7, 7, 12 and 10% of the grain weight, and the remaining cores to 60% of the total grain weights of the lines. No significant differences in the concentrations of nitrogen in the fractions were observed between the two RNAi lines (SE-1R and SE-10R), or between the RNAi lines and the null (SE-3N) and non-transgenic control (SE-24WT) lines (Supplementary Figure 5a). However, the total concentration of free amino acids determined by HPLC was higher in RNAi line SE-1R (Supplementary Figure 5b), which had the highest level of RNA suppression (Figure 3a).

Whole grain and pearling samples from RNAi SE-1R and the corresponding null line (SE-3N) were therefore analyzed for polar metabolites (which include 11 amino acids) using ¹H-NMR spectroscopy (Supplementary Table S3a,b). Supervised multivariate analysis (OPLS-DA) clearly separated the wholegrain flours from the SE-1 and SE-3 lines (Figure 4a), with the score contribution (Figure 4b) showing that the majority of metabolites were significantly elevated in SE-1R ($P < 0.05$, F-test): glutamine by 87%, asparagine by 57%, glycine by 50%, maltose by 279%, and glucose by 105% (Supplementary Table S3a,b). The compositions of the pearling fractions from the two lines were compared by PCA (Figure 4c). The different fractions are separated in PC1, which accounts for 75% of the total variation, while the two lines are separated in PC2, which accounts for 14% of the total variation. The loading plot for PC1 (Figure 4d) shows that the concentrations of all metabolites decreased from the bran (F1) to the core in both lines (SE-1 and SE-3), except for maltose which increased from F1 to F5 (Supplementary Figure 5c, Supplementary Table S3a, b). The loading plot for PC2 shows that amino acids accumulated to higher concentrations in SE-1R than in SE-3N, with glutamine, aspartate,

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394 and glycine being increased by 16%-52%, 29.9-41.8%, 21-62%, respectively, in the
395 different fractions. The results therefore demonstrated that the concentrations of most of
396 the amino acids transported by TaAAP13 were elevated by the suppression of TaAAP13
397 in line SE-1R.

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399 SDS-PAGE of total protein extracts showed that the total content of gluten proteins was
400 significantly reduced only in one RNAi line (SE-1R) (Supplementary Figure S6a, b), where
401 it was associated with a reduced proportion of ω -gliadins and increased proportion of
402 other gliadins (Supplementary Figure S6c).

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403 404 **Overexpression of TaAAP13 increases grain nitrogen content and grain size**

405 To determine whether overexpression of TaAAP13 can increase nitrogen accumulation
406 in wheat grain, transgenic plants were generated using TaAAP13 driven by two
407 promoters: the starchy-endosperm specific wheat high molecular weight glutenin subunit
408 (HMW-GS) 1Dx5 promoter and the constitutive maize ubiquitin promoter. Expression
409 under the control of the ubiquitin promoter did not affect grain nitrogen content
410 (Supplementary Figure S6a). By contrast, expression under the control of the HMW-GS
411 promoter significantly increased grain nitrogen concentration, grain size, thousand grain
412 weight, and nitrogen content per grain (Figure 5).

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414 With this promoter, the TaAAP13 expression levels in 21DPA caryopses were increased
415 9-12 fold in transgenic lines P16-OE and P22-OE (containing 6 copies) and by 30-50 fold
416 in lines P23-OE (28 copies), P24-OE, P25-OE and P26-OE (12-16 copies) compared with
417 a null line (P15-null) and a non-transgenic line (Cadenza, P29-WT) (Figure 5a). These six
418 over-expression lines all had increased concentrations of grain nitrogen compared to the
419 null and control lines, with statistically significant increases of 14.4% to 32.4% in P23-OE,
420 P24-OE and P26-OE (Figure 5b). The thousand grain weight (TGW), N content per grain
421 and grain size were also significantly increased by 19.3-31.7%, 31.2-72.3% and 9.3-
422 34.7% ($P < 0.05$, F-test), respectively, in P16-OE, P23-OE, P24-OE, and P26-OE (Figure
423 5c, 5d, Supplementary Table S4). However, the grain numbers per plant, grain yields per
424 plant and plant biomass were significantly decreased by 20.4 and 71.0%, 9.1 and 62%,
425 4.3% and 40.9% ($P < 0.05$, F-test) respectively, in the overexpression lines (P23-OE and
426 P24-OE) (Supplementary Figure S7).

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428 The grains were longer and wider, but more wrinkled in the overexpression lines with
429 higher transgene copy numbers and higher expression of TaAAP13 (Figure 5f). To
430 determine if the shriveled grains resulted from reduced accumulation of starch, three
431 overexpression lines (P26-OE, P23-OE, and P24-OE) with significantly increased
432 nitrogen concentrations in grains were selected and their total starch contents compared
433 with P15-null and non-transgenic lines (P29-WT) (Figure 5e). The results showed that
434 overexpression of TaAAP13 resulted in lower concentrations of total starch by 6.6-14.3%
435 and by 9.8-17.3% compared to the null and non-transgenic lines, respectively, but did not
436 affect the starch content per grain (Supplementary Table S5). To determine whether the

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distribution of protein and starch was altered by overexpression of TaAAP13, thin sections of whole mature grains were observed by light microscopy. This showed some protein bodies were fused to form a larger matrix in the sub-aleurone cells of the dorsal and lobe regions of overexpression line P23-OE with less starch granules compared to P15-null line (Figure 6), indicating that overexpression of TAAAP13 also affected the distribution of protein and starch in the starchy endosperm. SDS-PAGE showed that overexpression of TaAAP13 increased ω -gliadins and other gliadins (Supplementary Figure S8a), with a significant increase in the proportion of ω -gliadins in three lines and a significant decrease in the proportion of HMW subunits in two of the lines (Supplementary Figure S8b).

Determination of the profiles of polar metabolites from wholegrain flours using $^1\text{H-NMR}$ spectroscopy showed that the concentrations of 13 of the 15 free amino acids that were determined, including glutamine, proline and aspartic acid which were most abundant, were increased in the overexpression lines (P23-OE and P24-OE), by 1.5 to 2.2 fold compared with the null and wild type lines (Supplementary Figure S9). Increases were also observed in the most abundant soluble sugars, glucose, fructose and sucrose. The overexpression of TaAAP13 therefore supported the results of the yeast complementation, suggesting an ability to transport a broad range of amino acids in planta. Partial least squares discriminant analysis (PLS-DA) of 28 metabolites (including 15 amino acids) showed that the first two X-variates (accounting for 72.8% of the explained variation) separated the six overexpression lines from the P15-null line and the non-transgenic P29-WT line (Cadenza) (Supplementary Figure S10a). In particular, the control lines are positively associated with X-variate 1 and negatively associated with X-variate 2. These X-variates are defined by their loadings, shown in Supplementary Figure S10b. The results indicate that overexpression of TaAAP13 also changed the profiles of metabolites in the grains by accumulation of more free amino acids and soluble sugars in transgenic lines compared to the null or wild type lines.

Discussion

Nutrients (amino acids, sucrose, and monosaccharides) are transported from the vascular bundle in the crease into transfer cells in the nucellar projection, where they are released into the endosperm cavity (Wang *et al.*, 1994b), and subsequently taken up by the endosperm transfer cells (as shown schematically in Figure 7). The endosperm transfer cells are highly specialized cells with secondary wall ingrowth, which can amplify the membrane area up to 20-fold at 25 DPA and consequently enhance the efficiency and capacity of transport of solutes (Wang *et al.*, 1994a, Wang, *et al.*, 1995a). In this study, TaAAP2 was highly expressed in the endosperm transfer cells (ETC) during grain filling (14-28 DPA), which is associated with increased ingrowth of the endosperm transfer cell walls (Zheng and Wang, 2011) and increased protein synthesis in the starchy endosperm (Shewry *et al.*, 2012). Yeast complementation confirmed that TaAAP2 could transport a broad range of neutral amino acids, which was consistent with previous results (Fischer *et al.*, 1995, 2002; Okumoto *et al.*, 2002; Rentsch *et al.*, 2007). In addition, most

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AAPs are located in the plasma membrane (Okumoto *et al.*, 2004) and energized by H⁺ symporter (De Jong and Borstlap, 2000). Therefore, TaAAP2 expression may be coordinated with increased membrane surface area in the endosperm transfer cells to regulate the amino acid uptake rate to meet the demand of grain protein synthesis (Figure 7).

The embryo is isolated symplastically from the endosperm, and the epithelium cells of the scutellum function as transfer cells (Negbi, 1984) that can transport amino acids from the apoplast into the embryo (Figure 7b). The aleurone layer has high concentrations of vitamins, minerals, proteins and lipids (Geisler-Lee and Gallie, 2005). The differentiation of aleurone cells is initiated at 6-8 DPA and the accumulation of proteins and minerals occurs between about 11-27 DPA (Gillies *et al.*, 2012; Xiong *et al.*, 2013). TaAAP21 was most strongly expressed in the aleurone and scutellar epithelium between 14-28 DPA, and can complement many amino acids in yeast mutants, implying a role in transport of amino acids into the embryo and aleurone (Figure 7). This is supported by data on the *Arabidopsis* ortholog, AtAAP1, which is expressed in the epidermal transfer cells of the embryo and endosperm, thereby facilitating the import of amino acids into the embryo (Sanders *et al.*, 2009). However, the functions of TaAAP2 and TaAAP21 in transporting amino acids in the wheat grain need to be confirmed by direct functional analysis.

Amino acids are transported into the starchy endosperm for storage protein synthesis, and endosperm protein content is dependent on amino acid availability. TaAAP13 was expressed in the lobes of the starchy endosperm during early endosperm development, expressed more strongly in the sub-aleurone and inner starchy endosperm during the middle phase of grain development, but restricted to the sub-aleurone cells during late grain development. The highest level of expression of TaAAP13 in wheat endosperm was at 14 DPA at the start of the grain filling period and about a week before the maximum rate of protein accumulation (at about 21 DPA) (Wan *et al.*, 2008, 2013, 2014). ω -gliadins, α -gliadins, and low molecular weight subunits of glutenin are mainly deposited in the sub-aleurone cells and show a gradient from the outer cell layers to the inner endosperm determined by RNA *in-situ* hybridization, promoter::GUS and protein immunofluorescence (Van Herpen *et al.*, 2008; Tosi *et al.*, 2011; Wan *et al.*, 2014). TaAAP13 showed similar expression patterns and localization to storage proteins.

TaAAP13 can transport 15 amino acids by yeast complementation, notably, glutamine, which comprises 50–60% of the free amino acids in the wheat caryopses at 7 DPA (Howarth *et al.*, 2008) and is present in the endosperm cavity (43% of the free amino acid pool) at 21 DPA (Fisher and Macnicol, 1986). In addition, glutamine accounts for 30 and 50% of wheat amino acid residues in the gluten proteins stored in the wheat starchy endosperm (Shewry and Halford, 2002). This suggests that glutamine availability is very important for gluten protein synthesis.

Suppression of TaAAP13 expression did not reduce the total concentration of nitrogen in the mature grain or the radial distribution shown by pearling, but did result in increased concentrations of free amino acids (especially glutamine, glycine and asparagine) in both

Deleted: Two main factors determine grain protein content (GPC), (i) the loading of amino acids into the phloem in the vegetative tissues and (ii) the import of amino acids from the phloem into the grain. Amino acids are unloaded from the phloem via the vascular bundle into the endosperm cavity of the grain, where they are actively taken up by transfer cells; this is the first bottleneck for nutrient entry into the endosperm (Figure 7). Subsequently, amino acids are transported into the starchy endosperm where they are utilized for protein synthesis. The epithelium of the scutellum also takes up nutrients from the apoplast to support embryo development and protein synthesis. All these processes depend on the activity of amino acid transporters.¶ We therefore determined the expression patterns of three highly expressed AAPs, TaAAP2, TaAAP13, and TaAAP21, by promoter::GUS expression, RNA-seq and QPCR. TaAAP2 was highly expressed in the transfer cells during grain filling (14-28 DPA) indicating a role in amino acid uptake into the endosperm (Figure 8).

Moved up [2]: Amino acids are unloaded from the phloem via the vascular bundle into the endosperm cavity of the grain, where they are actively taken up by transfer cells; this is the first bottleneck for nutrient entry into the endosperm (Figure 7). Subsequently, amino acids are transported into the starchy endosperm where they are utilized for protein synthesis. The epithelium of the scutellum also takes up nutrients from the apoplast to support embryo development and protein synthesis. All these

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wholegrain flour and pearling fractions. A similar effect on total free amino acids was also observed in UMAMIT 11, 14, 28 and 29 loss-of-function mutants of Arabidopsis (Müller *et al.*, 2015). The concentrations of amino acids were also increased by more in the inner starchy endosperm and core fractions produced by pearling.

The amino acids taken up by endosperm transfer cells are transported across the starchy endosperm tissue to the sub-aleurone cells via a coordination of symplastic and apoplastic routes (De Jong and Borstlap, 2000; Wang, *et al.*, 1995a). However, there is no direct evidence to prove the cellular pathway of amino acid transport in wheat starchy endosperm. Ugalde and Jenner (1990) reported that the amino acid concentrations of dorsal endosperm showed a decrease from the endosperm cavity to the midpoint due to the influence of high concentrated fluid in endosperm cavity, followed by an increase from the midpoint to the periphery. However, in this study, the concentrations of 11 free amino acids increased from the endosperm core to the aleurone cells (present in pearling fraction F2) in both the RNAi and null lines (Supplementary Table S3a), indicating that amino acid transporters are required to transport nitrogen to the outer endosperm against gradients in amino acid concentrations. Most known Arabidopsis AAPs are active, proton-coupled amino acid symporters (Fischer *et al.*, 1995; Lohaus and Fischer, 2002; Tegeder and Ward, 2012). We therefore suggest that suppression of TaAAP13 expression reduced the import of the free amino acids (glutamine, glycine, and asparagine) into the sub-aleurone cells, which subsequently resulted in greater accumulation of free amino acids in the inner endosperm cells in the RNAi line compared to the null line (Figure 7). The repression of AtAAP1 function in seed loading Arabidopsis clearly decreases N import into the embryo, which in turn leads to an increase in the free amino acid pool in the pericarp, endosperm and most probably also in the seed apoplast (Sanders *et al.*, 2009). However, the concentrations of amino acids were also increased in mature grains of lines with overexpression of TaAAP13 under the control of HMW-GS. This suggests that amino acid transport capacity was increased by overexpressing TaAAP13, with more total nitrogen accumulation in individual grains. Free amino acids were increased both in RNAi and overexpression lines of TaAAP13, but the patterns of accumulation are different.

The overexpression of TaAAP13 not only increased the nitrogen concentration and the nitrogen content per grain, but also increased the concentration of gluten proteins, particularly ω -gliadins. These results suggest that TaAAP13 may play a role in importing amino acids into the endosperm for storage protein synthesis during the middle and late grain filling stages. The ectopic expression of HvSUT in wheat endosperm and VfAAP1 in pea cotyledons resulted in increased accumulation of gliadins and globulins in seeds respectively (Rolletschek *et al.*, 2005; Weichert *et al.*, 2010). By contrast, the proportion of ω -gliadins was reduced in the TaAAP13 RNAi line. The greater effect of TaAAP13 on ω -gliadins is consistent with the ω -gliadins being highly responsive to the nitrogen status (Wan *et al.*, 2014).

Deleted: However, the wheat TaAAP2 is unable to transport glutamine in the yeast complementation assay, indicating that the other amino acid transporters must be responsible for glutamine uptake in transfer cells, and its function *in planta* for amino acid transport needs to be further investigated. In contrast, both TaAAP13 and TaAAP21 transport glutamine and other amino acids. The RNAi and overexpression of TaAAP13 confirmed function in transport of glutamine, proline, glycine and other amino acids in wheat. ¶

The higher thousand grain weight (TGW) and larger grain size in the OE lines resulted mainly from the increased grain protein content. Although the starch concentration was decreased, the starch content per individual grain was not reduced. The remobilization and translocation of nitrogen to spikes strongly affects grain number (Santiago and Tegeder, 2016) and TGW and grain size showed both negative relationship with grain number per plant in the OE lines. This suggests that the increased sink capacity of the OE grain resulted in the redistribution of the limited nutrient supply into a smaller number of larger grains. Previous studies have shown that TGW and grain size were greatly increased by ectopic expression of barley sucrose transporter (HvSUT) in wheat grain (Weichert *et al.*, 2010) and of VfAAP1 in peas (Rolletschek *et al.*, 2005), with grain number per spike being decreased in wheat. Grain size is associated with the numbers and size of cells in the grain. Expressing the Arabidopsis phloem-specific sucrose transporter (AtSUC2) in rice phloem increased grain size, which resulted mainly from an increase in cell size and not cell number for large endosperm (Wang *et al.*, 2015). In TaAAP13 OE grains, more large cells filled with protein matrix were observed in the sub-aleurone, which may have contributed to the increased grain size. Unexpectedly, the plant biomass decreased in the OE lines, and it is possible that the strong sink for nitrogen in the endosperm could have affected embryo development by reducing the nutrient supply, which consequently had a negative impact on plant biomass. In contrast with the OE lines, no effects of TaAAP13 on TGW, total grain nitrogen content or grain size were observed in the RNAi lines. This may be because the RNAi suppressed TaAAP13 expression in whole plants, not only in the grain. However, more work is required to further investigate the mechanisms determining increased grain size, nitrogen accumulation, and their impact on plant biomass of OE lines in the future.

The study therefore suggests that overexpression of TaAAP13 in the starchy endosperm increased sink strength and hence grain size and weight by increasing the nitrogen uptake capacity of the grain. However, this resulted in reduced grain number due to limited availability of assimilate. This suggests that increases in grain nitrogen content and grain yield would require simultaneous increases in remobilized nitrogen to spike and in importing nitrogen in grain for future wheat breeding

Experimental procedures:

Expression analysis by RNA seq. Expression data for three amino acid transporters were extracted from RNA-seq data (Wan *et al.*, 2017; Pfeifer *et al.*, 2014; Choulet *et al.*, 2014). Expression units are in FPKM (frequency per kilobase million).

Harvest of materials and RNA extraction. The wheat cultivar Hereward was grown in field trials at Rothamsted Research in 2015, with either 200 kg N/ha fertilizer nitrogen or no nitrogen application. For the 200 kg N application, 50, 100, and 50 kg N/ha nitrogen (as ammonium nitrate) were applied at tillering, stem extension, and flag leaf emergence stages, respectively. Whole caryopses were harvested at 5, 10, 14, 17, 21, and 28 DPA

Deleted: Several published studies have demonstrated the role of AATs in developing seeds and fruit, using RNAi and loss-of-function mutants. For example, *ataap1* mutants decrease nitrogen import into the embryo, which in turn leads to the accumulation of large amounts of free amino acids in the seed coat and endosperm (Sanders *et al.*, 2009). Suppression of the expression of OsAAP6 in RNAi lines of rice also significantly reduced the grain protein content (Peng *et al.*, 2014). Loss-of-function mutants of four amino acid transporters (*umami1*, 14, 28 and 29) accumulate high levels of free amino acids in fruits and produce small seeds (Müller *et al.*, 2015). The *umami24* and *umami25* knockout lines decreased transfer of amino acids from the pericarp and endosperm to the embryo at 14 DPA, but recovered in the mature seeds without any deleterious effect on yield (Besnard *et al.*, 2018). In the present study, the total nitrogen concentration and gradients in mature grains of the TaAAP13 RNAi line was not significantly different from null and wild type lines, but large amounts of free amino acids (especially glutamine, glycine and asparagine) were significantly elevated in wholegrain flour and in pearling fractions. The concentrations of these amino acids were increased by 36-62% in fraction F5 and in the core. These results demonstrated that the import of the free amino acids (glutamine, glycine, and asparagine) transported by TaAAP13 into the sub-aleurone cells was decreased by suppression of TaAAP13 expression, which in turn may result in a high level of free amino acid accumulation in the inner endosperm cells in the RNAi line compared to null or wild type lines (Figure 7). ¶

Several attempts have been made to use the overexpression of amino acid transporter genes to increase nitrogen use efficiency, grain yield, and grain nitrogen content (Peng *et al.*, 2014; Rolletschek *et al.*, 2005). Targeted overexpression of a yeast SMM (S-methylmethionine) transporter (S-methylmethionine permease1, MMP1) in the phloem of pea increased phloem S and N loading and seed number (by 20%-27%), but did not increase seed weight (Tan *et al.*, 2010). By contrast, seed protein concentration and seed size were increased by 10-25% and 20-30%, respectively, when VfAAP1 was overexpressed in the storage parenchyma of cotyledons of pea and *Vicia narbonensis* under the control of the legumin B4 promoter (Rolletschek *et al.*, 2005). Simultaneous overexpression of PsAAP1 in the phloem and embryos of pea plants improved source-to-sink allocation of amino acids and led to up to 42% increased seed yield in seed number/plant and 23-33% in seed weight/plant. Seed storage protein concentrations were increased by 15-20%, which significantly increased N use efficiency in plants grown under both N deficiency and highly abundant N supply (Perchlik and Tegeder, 2017; Zhang *et al.*, 2015). In a reverse genetic study, *ataap8* mutants had reduced seed number (by 50%) resulting from decreased amino acid phloem loading and partitioning to sinks (Schmidt *et al.*, 2007; Santiago and Tegeder, 2016). These studies indicate that the concentrations of nitrogen in the phloem mainly

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898 (days post anthesis), and roots and leaves and stems at Zadoks 23 (2–3 tillers stage),
899 Zadoks 45 (booting stage), and 14 DPA and stored at -80°C for subsequent RNA
900 extraction and real-time PCR.

901 RNA extraction was modified as described by (Chang *et al.*, 1993). Frozen tissues were
902 ground in liquid nitrogen and extracted in CTAB buffer (2% (w/v) CTAB, 2% (w/v) PVP
903 K30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/l spermidine, 2% (w/v)
904 β -mercaptoethanol). The supernatant was extracted twice with chloroform:IAA (24:1) to
905 remove proteins. RNA was precipitated by addition of 0.25 volumes of 10 M LiCl and
906 incubation on ice overnight. The RNA pellet was dissolved in SSTE buffer (1.0 M NaCl,
907 0.5% (w/v) SDS, 10 mM Tris HCl pH 8.0, 1 mM EDTA) to remove polysaccharides and
908 extracted once with chloroform:IAA. After ethanol precipitation, total RNA was dissolved
909 in DEPC-treated water and stored at -80°C .

910 Total RNA was treated with DNAase to remove genomic DNA contamination and purified
911 through RNeasy mini spin columns (Qiagen). Two μg of RNA was used for reverse
912 transcription with SuperScriptTM III reverse transcriptase (Invitrogen) to synthesize cDNA
913 using anchored oligo (dT) 23 primers (Sigma-Aldrich).

914 **Real time PCR.** Real time PCR was performed using an ABI7500 (Applied Biosystems)
915 thermocycler. cDNA diluted 1:5 was used for RT-qPCR in a 25 μl reaction with SYBR @
916 Green JumpStartTM Taq ReadyMix (Sigma-Aldrich).

917 The Cell division control protein, AAA-superfamily ATPases (TraesCS4A02G035500) was
918 used as an internal control gene as it showed the most stable expression across different
919 wheat tissues and developmental stages (Paolacci *et al.*, 2009). The primers designed
920 for RT-qPCR are shown in Supplementary Table S1. For each pair of primers, PCR
921 efficiency was calculated in each run from a pool of all available cDNAs by using the
922 LinRegPCR software⁷⁹. All time points had three biological replicates. Normalised
923 relative quantity (NRQ) was calculated by CT values and primer efficiency (E) of the target
924 gene (T) in relation to the internal control gene (N) based on Rieu and Powers (2009) as
925 following formula: $\text{NRQ} = 1000 \times (E_{(T)})^{-\text{CT},T} / (E_{(N)})^{-\text{CT},N}$.

926 **Plasmid vector construction.** For promoter::GUS constructs, 1.412, 1.582 and 1.178
927 kb promoter fragments of TaAAP2B (B-genome), TaAAP13D (D-genome), and
928 TaAAP21A (A-genome) were amplified from wheat leaf genomic DNA and cloned into
929 pGEM-T Easy vector (Sigma), and then subcloned into vector pRRes104.293 containing
930 β -glucuronidase (GUS) with PmeI and NcoI restriction sites. For the RNAi plasmid
931 construct of TaAAP13, a 538bp fragment (125 to 662bp from ATG) of the coding region
932 was cloned into pGEM-T Easy vector, then subcloned into the RNAi cassette in
933 pRRes104RR.132 which contains with two pairs of restriction enzyme sites of BglII/BsrGI
934 and BamHI/BsiWI surrounding the maize ADH2 (alcohol dehydrogenase) intron.
935 Expression is driven by the maize ubiquitin promoter plus intron and Nos terminator. For
936 overexpression lines of TaAAP13, its full length of coding region was subcloned into
937 vector pRRes104RR.161 under the control of the wheat endosperm-specific DX5 HMW-

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939 GS promoter or vector pRRes104RR.125 under the control of the constitutive maize
940 ubiquitin promoter plus intron and Nos terminator.

941 **GUS assay.** Fresh grains at 7, 14, 21, 28 and 33 DPA were cut transversely or
942 longitudinally and incubated in staining buffer (1 mM X-Gluc, 100 mM sodium phosphate
943 pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 2% Triton X-
944 100) at 37°C for two hours to overnight. Roots, leaves and stems were vacuum-infiltrated
945 for 5 minutes before incubation in staining buffer. GUS-stained tissues were visualized
946 directly or after de-staining with ethanol and photographed using a Leica MA250 camera.

947 **Wheat Transformation.** The wheat cultivar Cadenza was transformed using particle
948 bombardment to deliver plasmid DNAs into immature embryos and transgenic plants
949 regenerated via somatic embryogenesis (Sparks and Jones, 2014). Transgenic plants
950 were confirmed by PCR using gene-specific primers. Transformed plants were grown to
951 maturity in the GM glasshouse.

952 **Homozygous assay and growth of transgenic plants.** DNA was extracted from leaves
953 of T1 seedlings and used for determination of homozygosity and transgene copy numbers
954 by iDna Genetics Ltd (Norwich Research Park, UK). Wheat plants were grown in a GM
955 glasshouse with 16 hours day at 20°C and 8 hours night at 15°C. The plants were watered
956 once a day using a flood bench system lined with capillary matting. Four biological
957 replicates comprising 4 plants each were grown in a randomized order.

958 **Complementation of yeast amino acid transporter mutants.** Full length coding
959 regions of TaAAP2, TaAAP13 and TaAAP21 were PCR amplified from cDNA of 21 DPA
960 wheat grains, cloned into pGEM-T Easy vector and confirmed by sequencing. The three
961 AAP genes were subcloned into vector PDR196 between a PMA (plasma membrane H⁺-
962 ATPase) promoter and ADH terminator, and *Saccharomyces cerevisiae* strains 22Δ8AA,
963 22 Δ6AAL, YDR544.137, 30.537a, 21.983c, and 22574d were transformed according to
964 Dohmen *et al.*, 1991. The transformants, negative control (empty vector) and positive
965 controls (containing Arabidopsis AAPs) were selected on nitrogen-free media containing
966 1g/l for proline, aspartate, glutamate and GABA, 1mM for citrulline, glycine, Isoleucine,
967 methionine, phenylalanine, and valine, 5mM for leucine, threonine, tryptophan and
968 tyrosine, 0.5g/l for arginine, and 0.1mM for lysine with 1g/l Urea. For non-selective
969 conditions, media were supplemented with 0.5g/l ammonium sulfate and grown at 30°C
970 for 5-7 days (Fischer *et al.*, 2002).

971 **Nitrogen determination.** Total nitrogen was determined using the American Society for
972 Testing and Materials (ASTM) standard protocol E1019 using a Leco combustion analysis
973 system based on the Dumas method. 150 mg of milled wheat grain and four biological
974 replicas were analyzed.

975 **Pearling of grains.** 20g mature seeds were pearled in a Streckel & Schrader (Hamburg,
976 Germany) pearling mill (based on Tosi *et al.*, 2011). Five fractions were prepared by
977 sequential pearling, corresponding to about 4, 7, 7, 12 and 10 % of the grain weight, and
978 the remaining cores (60%) and whole grains were milled in a centrifugal mill (Retsch,

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10 980 ZM200). These pearly fractions are enriched in embryo and pericarp tissue (F1),
11 981 aleurone layer (F2), sub-aleurone layer (F3) and two progressively more central areas of
12 982 the starchy endosperm (F4 and F5), respectively. Three biological replicates were
13 983 performed.

14 984 Grain area, length, width, moisture and biomass measurement. Seed parameters of
15 985 area (size), length and width were measured using MARVIN- Digital Seed Analyzer SN
16 986 176 (Marvitech - Germany). Four biological replicates from 16 plants (200-400 grains from
17 987 each biological replicate) were analyzed. Seed moisture was detected using Bruker
18 988 Minispec mq20 TD-NMR Contrast Agent analyzer (Germany) with bespoke robot and
19 989 recording software (ROHASYS, Dutch Robotics) by loading 7-10g seeds. Thousand grain
20 990 weight (TGW) was converted into 15% seed moisture grain weight. Biomass (above
21 991 ground stems and leaves) were oven dried at 80°C overnight.

22 992
23 993 **SDS-PAGE.** 10mg flour was suspended in 300µl total protein extraction buffer [50 mM
24 994 Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 2 % (w/v) dithiothreitol (DTT) and
25 995 0.1 % (w/v) bromophenol blue] (Wan *et al.*, 2014). The extracts were heated for 3 minutes
26 996 at 95°C, and centrifuged for 15 minutes. 3 µl of the supernatants were separated on pre-
27 997 cast 4–12 % Bis-Tris Nu-PAGE gels (Invitrogen). The four biological extracts, and two
28 998 technical gel running were repeated. The gels were stained with Coomassie BBR250 in
29 999 10 % (w/v) trichloroacetic acid (TCA), 40 % (v/v) methanol, and de-stained in 10 % (w/v)
30 1000 TCA. The gels were scanned with a HPG4010 scanner, and the images from grey tif files
31 1001 were processed with Phoretix 1D advanced software (Nonlinear Dynamics, Durham, NC,
32 1002 USA). Each group protein percentage is expressed as a % of total gluten proteins.

33 1003 **¹H-Nuclear magnetic resonance (NMR) spectroscopy.** ¹H-NMR sample preparation
34 1004 was carried out according to (Baker *et al.*, 2006). Wholegrain samples (30 mg) (three
35 1005 technical replicates each of three biological replicates) were extracted in 80:20
36 1006 D₂O:CD₃OD containing 0.05% d₄-trimethylsilylpropionate (TSP; 1mL) as an internal
37 1007 standard at 50°C for 10 min. After centrifugation (5 min at 13 000 rpm), the supernatant
38 1008 was removed and heated to 90°C for 2 min to halt enzyme activity. After cooling and
39 1009 further centrifugation, the supernatant (650 µL) was transferred to a 5 mm NMR tube for
40 1010 analysis. The data collection and analysis were described as in Shewry *et al.* (2017).

41 1011 **High performance liquid chromatography (HPLC).** Free amino acids were extracted
42 1012 according to Curtis *et al.* (2018). 30 mg samples (four biological replicates) were
43 1013 suspended in 500 µL of 0.01 N HCl by shaking for 30 min at room temperature. After
44 1014 centrifugation at 10000 rpm for 15 minutes, the supernatants were filtered through a 0.45
45 1015 µm poly (ether sulfone) filter before analysis. Amino acids were separated using a Waters
46 1016 Alliance 2795 HPLC system (Waters Corp., Milford, USA) coupled with a Waters 474
47 1017 scanning fluorescence detector. The detailed method is described in Shi *et al.* (2019).

48 1018 **Wheat grain fixation, embedding and light microscopy.** The middle parts of 5 mature
49 1019 grains were cut into 2 mm transverse sections and immediately fixed in 4 % (w/v)

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10 1022 paraformaldehyde in 0.1 M Sorenson's phosphate buffer (NaH₂PO₄·2H₂O and Na₂HPO₄·
11 1023 12H₂O, pH 7.0) with 2.5 % (w/v) glutaraldehyde overnight. After dehydration in increasing
12 1024 concentrations of ethanol, the sections were embedded in LR White Resin for two weeks
13 1025 at room temperature and polymerized at 55°C. A Reichert-Jung Ultracut ultramicrotome
14 1026 was used to section the resin-embedded grains at 0.5 µm or 1 µm thickness for protein
15 1027 staining. Protein bodies were stained with 1 % (w/v) Naphthol Blue Black in 7 % (w/v)
16 1028 acetic acid. The slides were visualized under a Zeiss Axiophot microscope and images
17 1029 were acquired with a RetigaExi CCD digital camera (Qimaging, Surrey, BC, Canada)
18 1030 under bright field optics and MetaMorph software version 7.5.5 9 (Molecular Devices,
19 1031 Sunnyvale, CA, USA).

20 1032 **Total starch assay.** The total starch contents of samples containing resistant starch
21 1033 were determined using the Megazyme total starch kit (amyloglucosidase/amylose method,
22 1034 Megazyme, Bray, Ireland). Wholegrain flour (100mg) with four biological replicates and
23 1035 two technical extracts was extracted with dimethyl sulphoxide, and digested with a
24 1036 thermostable alpha-amylase at 100°C for 15 minutes. Amyloglucosidase, was added to
25 1037 release glucose at 50°C for 30 minutes. The final glucose content was calculated after
26 1038 incubation with glucose oxidase/oxidase and absorbance measurement at 510 nm
27 1039 against blank and glucose standards

28 1040 **Statistical analysis.** Data were analyzed using ANOVA accounting for the randomized
29 1041 block design. Where necessary, data were log or square root transformed to satisfy
30 1042 homogeneity of variance. The least significant difference (LSD) values presented are the
31 1043 LSD associated with comparisons. Analyses were done using the GenStat (19th edition,
32 1044 VSN International Ltd., Hemel Hempstead, U.K.). Principal component analysis (PCA)
33 1045 and partial least squares discriminant analysis (PLS-DA) were applied using the
34 1046 correlation matrix between variables. Each input variable was transformed according to
35 1047 the univariate analysis, adjusted for any block effects and averaged over technical
36 1048 replicates. PCA was performed in Genstat, 19th edition. The software Simca-P v. 16
37 1049 (Sartorius Stedim Data Analytics AB) was used for OPLS-DA. The analysis was carried
38 1050 out using quantified 1H-NMR data scaled to unit variance.

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45 1057 **Conflict of interests**

46 1058 The authors declare that they have no competing interests.

48 1059 **Author contributions**

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10 1063 YFW, PRS, MJH conceived the conception and wrote the manuscript, YFW and PB
11 1064 contributed all the constructs, GUS expression, starch assay, YW and ZQS contributed
12 1065 to microscopy and protein work, DR performed yeast complementation, JW performed
13 1066 ¹H-NMR, CAS and AKH provided transgenic service and plasmid vector cassette, KH
14 1067 and SP analyzed the data. All the authors revised the manuscript for submission.

15 1068 **Supporting information:**

16 1069 Supplementary Figure S1. The expression patterns of TaAAP2, TaAAP13, and TaAAP21
17 1070 in wheat organs by RNA-seq.

18 1071 Supplementary Figure S2. The localization of TaAAP2, TaAAP13, and TaAAP21
19 1072 expression by promoter::GUS analysis.

20 1073 Supplementary Figure S3. Complementation of yeast mutants by TaAAP2, TaAAP13,
21 1074 and TaAAP21.

22 1075 Supplementary Figure 4: The agronomic traits of the RNAi lines (SE-1R, SE-9R, SE-
23 1076 10R), Nulls (SE-3N, SE-11N), and non-transgenic line (SE-24WT).

24 1077 Supplementary Figure S5, The nitrogen concentration and free amino acids in RNAi lines
25 1078 of wholegrain flour and pearling fractions.

26 1079 Supplementary Figure S6. The gluten protein and composition of wholegrain flour by
27 1080 SDS-PAGE in RNAi lines (SE-1R, 9R, 10R), nulls (SE-3N, 11N) and non-transgenic line
28 1081 (SE-24WT, Cadenza).

29 1082 Supplementary Figure S7. phenotypes of overexpression of TaAAP13 lines.

30 1083 Supplementary Figure S8. SDS-PAGE and protein composition of overexpression lines.

31 1084 Supplementary Figure S9. Metabolites of wholegrain flour from overexpression lines were
32 1085 determined by ¹H-NMR.

33 1086 Supplementary Figure S10. A Partial least squares discriminant analysis (PLS-DA) of
34 1087 metabolites from overexpression lines by ¹H-NMR.

35 1088 Supplementary Table S1. primers for RT-qPCR and plasmid vector constructs.

36 1089 Supplementary Table S2. The amino acids of TaAAP2, TaAAP13, and TaAAP21
37 1090 complementation in yeast mutants.

38 1091 Supplementary Table S3. The metabolite changes in RNAi line (SE-1) and Null (SE-3)
39 1092 whole grain flour and their pearling fractions by ¹H-NMR.

40 1093 Supplementary Table S4. The grain size of pHMW-TaAAP13 overexpression lines.

41 1094 Supplementary Table S5: The starch content (mg) /per grain in overexpression lines.

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1304 **Figure legends:**

1305 Figure 1: The expression patterns of TaAAP2, TaAAP13, and TaAAP21.

1306 (a-c). Expression of TaAAP2 (a), TaAAP13 (b), and TaAAP21 (c) in grain cells:
1307 endosperm transfer cells (ETC), starchy endosperm (SE), aleurone cells (AL) of Chinese
1308 Spring at 20DPA (days post anthesis) (Pfeifer et al 2014; Wan et al, 2017) by RNA-seq
1309 data. (d-f). Expression of TaAAP2 (d), TaAAP13 (e), and TaAAP21 (f) in different organs
1310 of wheat cultivar Hereward from Whole caryopses at 5, 10, 14, 17, 21, and 28 DPA (days
1311 post anthesis), roots, leaves and stems at Zadoks 23 (2–3 tillers stage) and Zadoks 45
1312 (booting stage) at two nitrogen levels 0kg N/ha and 200kg N/ha by RT-qPCR. Error bars
1313 represent standard errors (SE).

1314 Figure 2. Localization of TaAAP2B, TaAAP13D, and TaAAP21A by promoter::GUS
1315 expression.

1316 The GUS expression patterns driven by promoters of TaAAP2B, TaAAP13D, and
1317 TaAAP21A in T2 developing grains at 7, 14, 21, 28 and 33DPA (days post anthesis). The
1318 GUS staining was visualised directly for TaAAP2B and TaAAP13D or after serial ethanol
1319 de-staining for TaAAP21A under stereomicroscope Leica MA250. Scale bars represent
1320 500 μm except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 μm . ETC:
1321 endosperm transfer cells, EM: embryo, AL: aleurone cells, ES: epithelium of scutellum,
1322 SE: starchy endosperm, RP: root primordia.

1323 Figure 3. TaAAP13 expression and nitrogen concentrations in RNAi lines (SE-1R, SE-
1324 9R, SE-10R), null lines (SE-3N, SE-11N), and non-transgenic line SE-24WT (Cadenza,
1325 wild type).

1326 (a). TaAAP13 gene expression at 14DPA of wheat developing grain. Asterisks (***)
1327 indicated the significant differences between RNAi lines and nulls or wild type detected
1328 by ANOVA ($P < 0.001$, F-test). Error bars represent standard errors. (b). Nitrogen
1329 concentrations of wholegrain four and white flour. The differences between RNAi lines
1330 and nulls were not statistically significant ($P < 0.05$, F-test).

1331 Figure 4. Metabolite data analysis of RNAi and null lines by $^1\text{H-NMR}$.

1332 (a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null
1333 SE-3N by $^1\text{H-NMR}$. (b). Contribution plot comparing significant component changes
1334 from two lines SE-1R and SE-3N; the red colour represents more elevated in SE-3N on
1335 the top, and in SE-1R at the bottom. The amino acids (glutamine and glycine) were most
1336 elevated in SE-1R, and are indicated by blue arrows. (c). PCA scores plot of pearling
1337 fractions: F1, F2, F3, F4, F5, core and wholegrain flour of SE-1R and SE-3N. (d). the
1338 loading plot of PCA for pearling fractions. The coloured circles were drawn by separation
1339 of different lines in (a) and different fractions in (c), rather than statistically significant. F1,
1340 F2, F3, F4, F5 and core are mainly enriched in bran (F1), aleurone cell (F2), sub-aleurone

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10 1342 cells (F3), two progressive inner endosperm fractions (F4, F5) and core. (d). Loading plot
11 1343 of (c).

12 1344 Figure 5. The gene expression and phenotype of overexpression TaAAP13 under the
13 1345 control of endosperm-specific promoter HMW-GS Dx5.

14 1346 (a). Gene expression of TaAAP13 at 21DPA in developing grains of overexpression lines:
15 1347 P22-OE and P16-OE (6 copies), P25-OE (16 copies), P26-OE (14copies), P23-OE (26
16 1348 copies), P24 (12 copies), null (P15-null), and non transgenic (P29-WT, Cadenza). (b). N
17 1349 concentration % of wholegrain flour. (c). Thousand grain weight (TGW) at 15% moisture
18 1350 content (g). (d). Nitrogen content (mg) per grain. (e). Total starch concentration in
19 1351 wholegrain flour. (f). Grain morphology. Scale bar represents 1 cm in (f). Significant
20 1352 differences were detected using ANOVA ($P < 0.05$, F-test) and indicated with asterisk
21 1353 (*), and ($P < 0.001$, F-test) with asterisk (***).

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22 1354 Figure 6. The protein distribution of mature grain resin sections of TaAAP13
23 1355 overexpression line.

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25 1356 (a). Dorsal of P23-OE. (b). Dorsal of P15-null. (c). Lobe of P23-OE. (d). Lobe of P15-null.
26 1357 (e). Whole grain section of P23-OE. (f). Whole grain section of P15-NULL. Resin sections
27 1358 with 1 μm thickness in (a)-(d) and 0.5 μm thickness in (e) and (f) were stained with 1 %
28 1359 (w/v) Naphthol Blue Black in 7 % (w/v) acetic acid. Scale bars represent 50 μm in (a)-(d)
29 1360 and 1mm in (e) and (f). Red arrows Indicated large protein body matrix (PB). AL: aleurone
30 1361 cells. SA: sub-aleurone cells. SG: starch granules.

31 1362 Figure 7. Schematic view of three TaAAPs nitrogen transport. (a). Wheat grain transverse
32 1363 section. (b) wheat grain longitudinal section. VB: vascular bundle. EC: endosperm cavity
33 1364 for amino acids delivered to endosperm from vascular bundle. NPTC: nucellar projection
34 1365 transfer cells. ETC: endosperm transfer cells. TaAAP2 expression localization for uptake
35 1366 of amino acids from endosperm cavity. SE: starch endosperm. SA: sub-aleurone cells for
36 1367 proteins (mainly ω and α -gliadins, LMW-glutenin subunits), and TaAAP13 expression
37 1368 localization. AL: aleurone cells for TaAAP21 expression localization. ES: epithelium of
38 1369 scutellum for TaAAP21 expression localization. SC: scutellum. EM: embryo. ↓ : TaAAP2.
39 1370 ↓ : TaAAP13. ↑ : TaAAP21. ● : protein bodies

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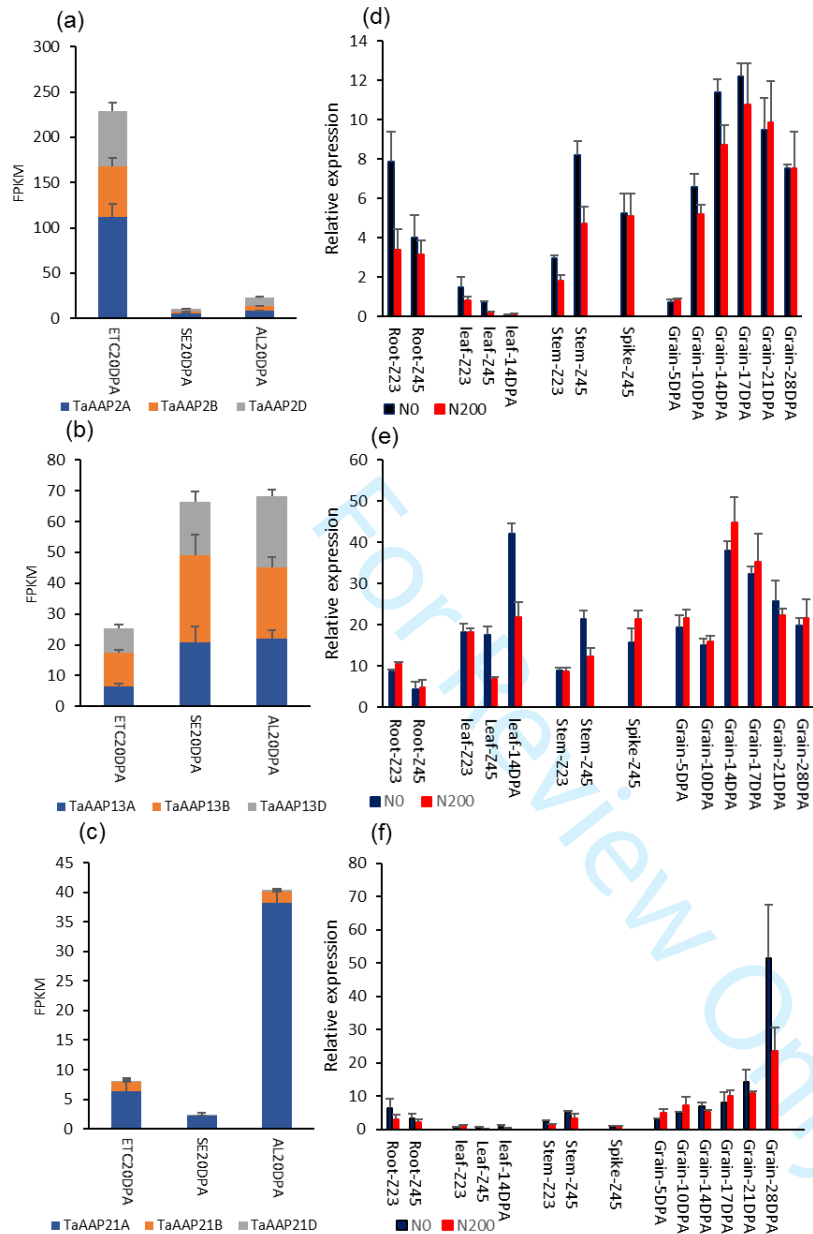


Figure 1: The expression patterns of TaAAP2, TaAAP13, and TaAAP21.

(a-c). Expression of TaAAP2 (a), TaAAP13 (b), and TaAAP21 (c) in grain cells: endosperm transfer cells (ETC), starchy endosperm (SE), aleurone cells (AL) of Chinese Spring at 20DPA (days post anthesis) (Pfeifer et al 2014; Wan et al, 2017) by RNA-seq data. (d-f). Expression of TaAAP2 (d), TaAAP13 (e), and TaAAP21 (f) in different organs of wheat cultivar Hereward from Whole caryopses at 5, 10, 14, 17, 21, and 28 DPA (days post anthesis), roots, leaves and stems at Zadoks 23 (2–3 tillers stage) and Zadoks 45 (booting stage) at two nitrogen levels 0 kgN/ha and 200kg N/ha by RT-qPCR. Error bars represent standard errors (SE).



Figure 2. Localization of TaAAP2B, TaAAP13D, and TaAAP21A by promoter::GUS expression.

The GUS expression patterns driven by promoters of TaAAP2B, TaAAP13D, and TaAAP21A in T2 developing grains at 7, 14, 21, 28 and 33DPA (days post anthesis). The GUS staining was visualised directly for TaAAP2B and TaAAP13D or after serial ethanol de-staining for TaAAP21A under stereomicroscope Leica MA250. Scale bars represent 500 μ m except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 μ m. ETC: endosperm transfer cells, EM: embryo, AL: aleurone cells, ES: epithelium of scutellum, SE: starchy endosperm, RP: root primordia.

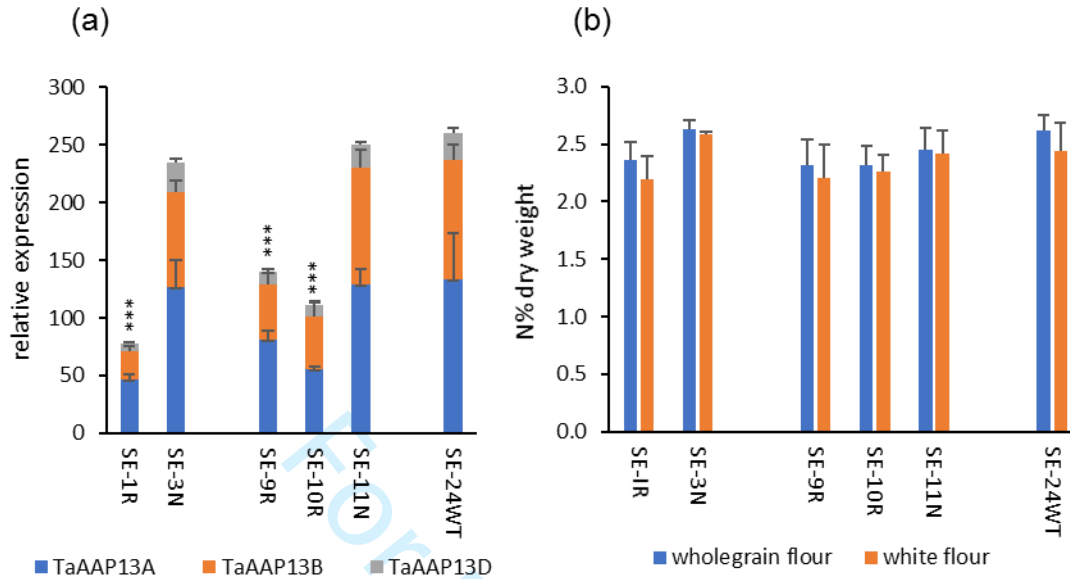


Figure 3. TaAAP13 expression and nitrogen concentrations in RNAi lines (SE-1R, SE-9R, SE-10R), null lines (SE-3N, SE-11N), and non-transgenic line SE-24WT (Cadenza, wild type).

(a). TaAAP13 gene expression at 14DPA of wheat developing grain. Asterisks (***) indicated the significant differences between RNAi lines and nulls or wild type detected by ANOVA ($P < 0.001$, F-test). Error bars represent standard errors. (b). Nitrogen concentrations of wholegrain flour and white flour. The differences between RNAi lines and nulls were not statistically significant ($P < 0.05$, F-test).

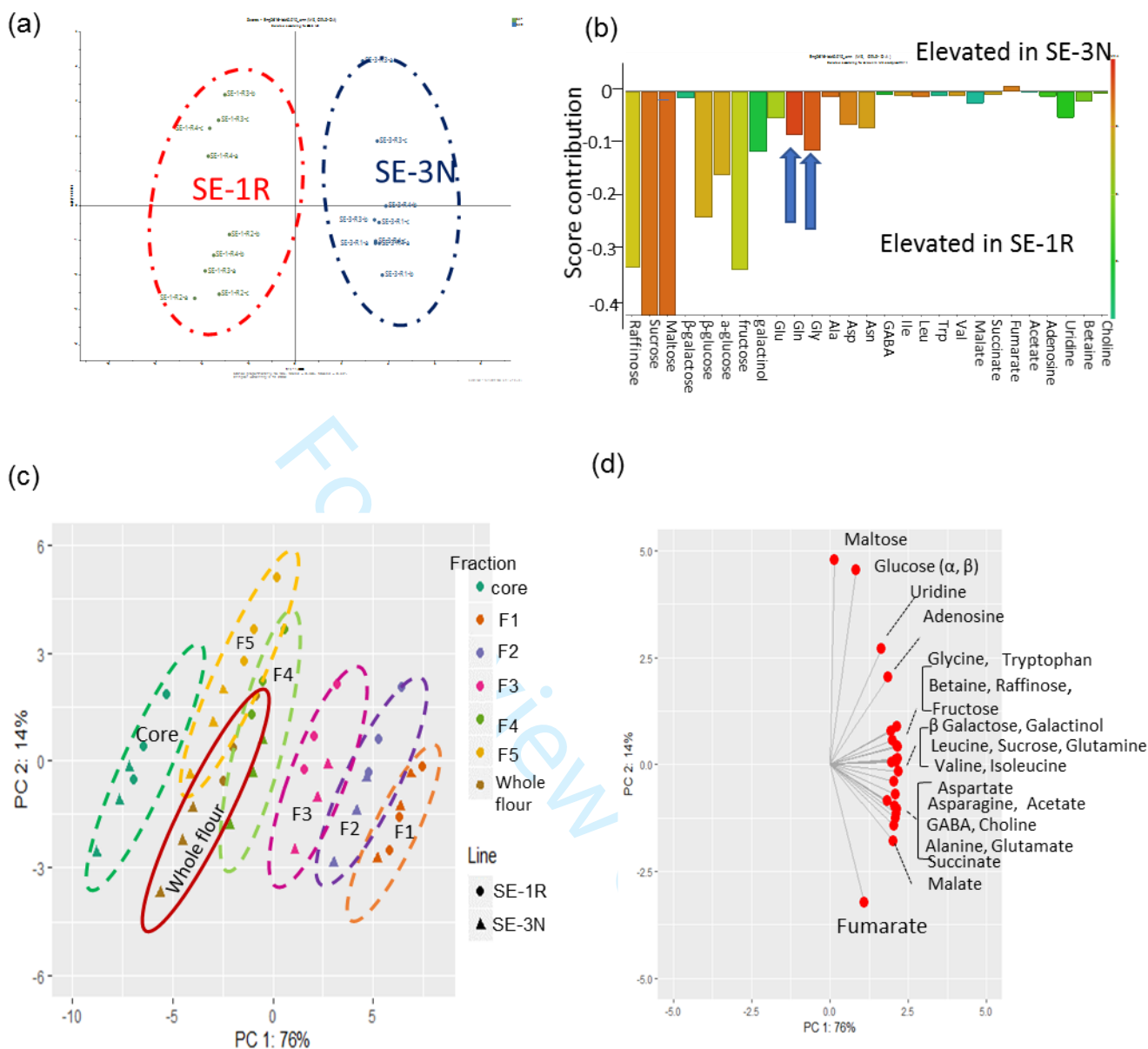


Figure 4. Metabolite data analysis of RNAi and null lines by $^1\text{H-NMR}$.

(a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null SE-3N by $^1\text{H-NMR}$. (b). Contribution plot comparing significant component changes from two lines SE-1R and SE-3N; the red colour represents more elevated in SE-3N on the top, and in SE-1R at the bottom. The amino acids (glutamine and glycine) were most elevated in SE-1R, and are indicated by blue arrows. (c). PCA scores plot of pearling fractions: F1, F2, F3, F4, F5, core and wholegrain flour of SE-1R and SE-3N. (d). the loading plot of PCA for pearling fractions. The coloured circles were drawn by separation of different lines in (a) and different fractions in (c), rather than statistically significant. F1, F2, F3, F4, F5 and core are mainly enriched in bran (F1), aleurone cell (F2), sub-aleurone cells (F3), two progressive inner endosperm fractions (F4, F5) and core. (d). Loading plot of (c).

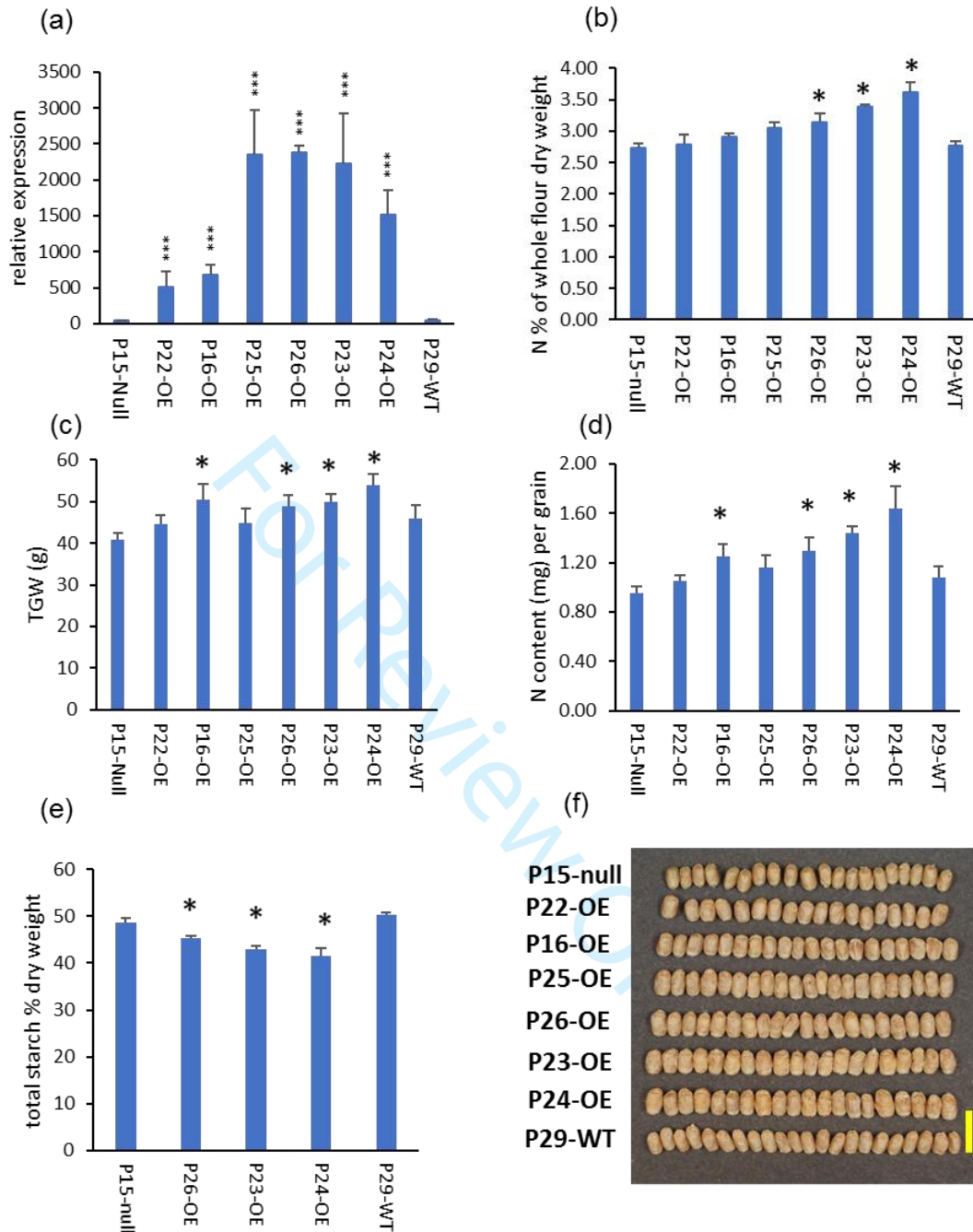


Figure 5. The gene expression and phenotype of overexpression TaAAP13 under the control of endosperm-specific promoter HMW-GS Dx5. (a). Gene expression of TaAAP13 at 21DPA in developing grains of overexpression lines: P22-OE and P16-OE (6 copies), P25-OE (16 copies), P26-OE (14copies), P23-OE (26 copies), P24 (12 copies), null (P15-null), and non transgenic (P29-WT, Cadenza). (b). N concentration % of wholegrain flour. (c). thousand grain weight at 15% moisture content (g). (d). Nitrogen content (mg) per grain. (e). Total starch concentration in wholegrain flour. (f). Grain morphology. Scale bar represents 1 cm in (f). Significant differences were detected using ANOVA ($P < 0.05$, F-test) and indicated with asterisk (*), and ($P < 0.001$, F-test) with asterisk (***)

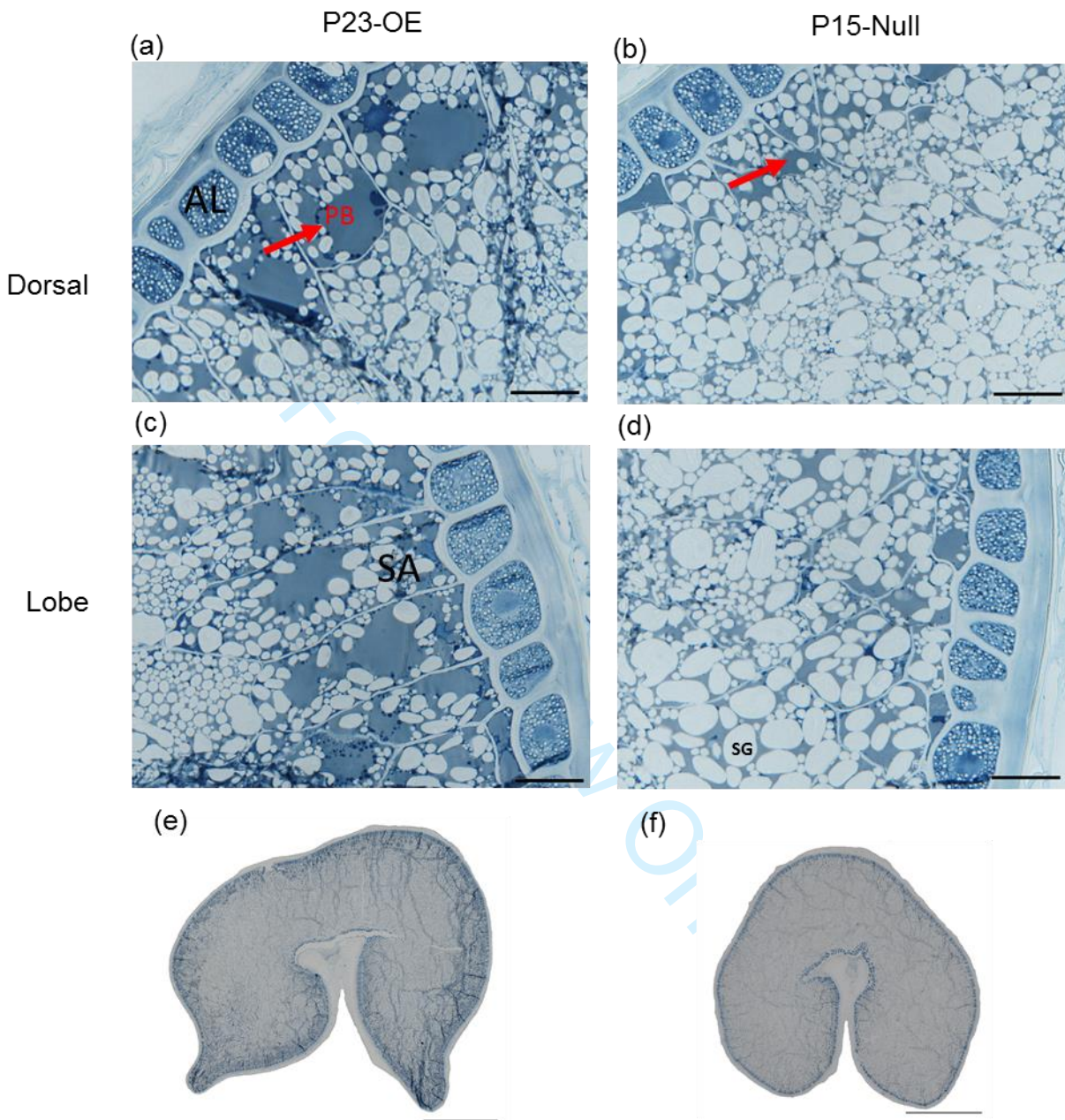


Figure 6. The protein distribution of mature grain resin sections of TaAAP13 overexpression line.

(a). Dorsal of P23-OE. (b). Dorsal of P15-null. (c). Lobe of P23-OE. (d). Lobe of P15-null. (e). Whole grain section of P23-OE. (f). Whole grain section of P15-NULL. Resin sections with 1 μm thickness in (a)-(d) and 0.5μm thickness in (e) and (f) were stained with 1 % (w/v) Naphthol Blue Black in 7 % (w/v) acetic acid. Scale bars represent 50 μm in (a)-(d) and 1mm in (e) and (f). Red arrows Indicated large protein body matrix (PB). AL: aleurone cells. SA: sub-aleurone cells. SG: starch granules.

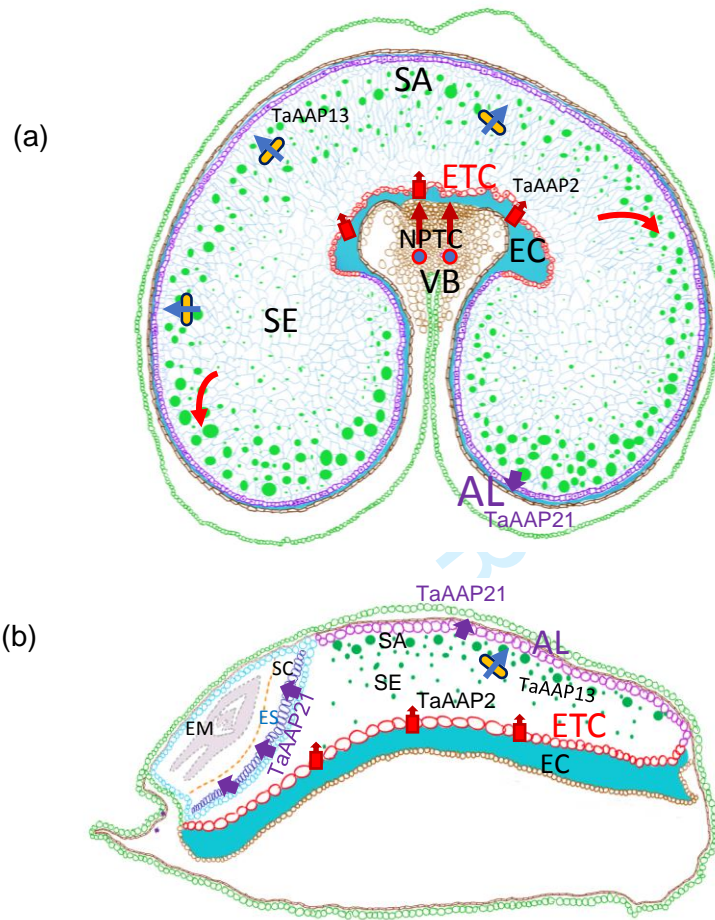






Figure 7. Schematic view of three TaAAPs nitrogen transport. (a). Wheat grain transverse section. (b) wheat grain longitudinal section. VB: vascular bundle. EC: endosperm cavity for amino acids delivered to endosperm from vascular bundle. ETC: endosperm transfer cells. TaAAP2 expression localisation for uptake of amino acids from endosperm cavity. SE: starch endosperm. SA: sub-aleurone cells for proteins (mainly ω - and α -gliadins, LMW-glutenin subunits), and TaAAP13 expression localisation. AL: aleurone cells for TaAAP21 expression localisation. ES: epithelium of scutellum for TaAAP21 expression localisation. SC: scutellum. EM: embryo. : TaAAP2. : TaAAP13. : TaAAP21. : protein bodies.