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

Wheat amino acid transporters highly expressed in grain cells regulate grain size 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.

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 (AAAP), 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), ^{1}H-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).

Introduction

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

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

Only a few AAT genes have been functionally characterized, mainly in Arabidopsis. Nevertheless, studies indicate that they play important roles in amino acid uptake into roots, phloem loading, long-distance transport and loading into the seed (Dinkeloo *et al.*, 2017; Tegeder, 2012). Crucial functions for the import of amino acids into sink tissues (seed, fruit, and tuber) have been described for different amino acid transporters. The AtAAP1 transporter is highly expressed in the embryo epithelium (transfer cells) of Arabidopsis and involved in uptake and transport of amino acids from the endosperm into the embryo, with *ataap*1 mutants having lower seed protein content and seed weight (Sanders *et al.*, 2009). RNAi-mediated down-regulation of the StAAP1 transporter can reduce the levels of free amino acids by 50% in potato tubers (Koch *et al.*, 2003). The tonoplast-localized UMAMIT24 of Arabidopsis is able to transport the amino acids temporarily stored in the vacuoles of chalaza cells before being delivered to filial tissue, while the plasma membrane-localized UMAMIT 25 is expressed in the endosperm and pericarp and could mediate amino acid export from endosperm (Besnard et al., 2018). The UMAMIT18 transporter is present in vascular tissues and developing seeds, supporting accumulation nitrogen in developing siliques (Ladwig *et al.*, 2012). UMAMIT11 and 14 AATs are expressed in the chalaza (unloading domain) of developing seeds and UMANIT28 and 29 transporters export amino acids from the endosperm and pericarp, respectively. Single loss-of-function mutants of these four transporters resulted in the accumulation of high levels of free amino acids in the seeds and greatly reduced seed size (Müller *et al.*, 2015). By contrast, overexpression of VfAAP1 (which is highly expressed in embryonic storage parenchyma cells at early maturation) in both pea and

Vicia narbonensis (Narbon bean) seeds resulted in increased seed protein content (by 10-25%) and seed size (by 20-30%), by increasing import of amino acids into the embryo (Rolletschek *et al.*, 2005). Similarly, the simultaneous overexpression of PsAAP1 in the phloem and embryos of pea plants increased seed yield and seed storage protein levels when the plants were grown with highly abundant N, due to increased source-to-sink allocation of amino acids (Zhang *et al.*, 2015) and increased nitrogen use efficiency (Perchlik and Tegeder, 2017). The OsAAP6 transporter, which is highly expressed in the endosperm of rice, functions as a positive regulator of GPC and overexpression in rice greatly increased GPC in rice grain when overexpressed (Peng *et al.*, 2014). Tomato SlCAT9 is a tonoplast Glu/Asp/GABA transporter, and overexpression of SlCAT9 greatly affects the flavor profile of the tomato fruit by increasing the accumulation of these amino acids during fruit development (Snowden *et al.*, 2015).

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

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

Results

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

Previously three TaAAP genes (TaAAP2, TaAAP13, and TaAAP21) out of 100 homoeologous groups of amino acid transporters (283 genes) were shown to be highly expressed in the endosperm transfer cells, starchy endosperm cells and aleurone cells of wheat grains, respectively (Wan *et al.*, 2017). These genes were therefore selected to determine their gene expression patterns, spatial tissue localization and potential functions in grain nitrogen metabolism.

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).

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

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

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

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

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

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 ^{1}H-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,

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

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

Overexpression of TaAAP13 increases grain nitrogen content and grain size

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

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

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

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}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

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 endopserm (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

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 TaAAAP13 on ω-gliadins is consistent with the ω-gliadins being highly responsive to the nitrogen status (Wan *et al.*, 2014).

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 mumber 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 determinining 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

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

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

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

Real time PCR. Real time PCR was performed using an ABI7500 (Applied Biosystems) thermocycler. cDNA diluted 1:5 was used for RT–qPCR in a 25 µl reaction with SYBR ® Green JumpStart ™ Taq ReadyMix (Sigma-Aldrich).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

paraformaldehyde in 0.1 M Sorenson's phosphate buffer ($NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$, pH 7.0) with 2.5 % (w/v) glutaraldehyde overnight. After dehydration in increasing concentrations of ethanol, the sections were embedded in LR White Resin for two weeks at room temperature and polymerized at 55°C. A Reichert-Jung Ultracut ultramicrotome was used to section the resin-embedded grains at 0.5 µm or 1 µm thickness for protein staining. Protein bodies were stained with 1 % (w/v) Naphthol Blue Black in 7 % (w/v) acetic acid. The slides were visualized under a Zeiss Axiophot microscope and images were acquired with a RetigaExi CCD digital camera (Qimaging, Surrey, BC, Canada) under bright field optics and MetaMorph software version 7.5.5 9 (Molecular Devices, Sunnyvale, CA, USA).

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

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

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Conflict of interests

The authors declare that they have no competing interests.

Author contributions

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

Supporting information:

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

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

Supplementary Figure S3. Complementation of yeast mutants by TaAAP2, TaAAP13, and TaAAP21.

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

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

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

Supplementary Figure S7. phenotypes of overexpression of TaAAP13 lines.

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

Supplementary Figure S9: Metabolites of wholegrain flour from overexpression lines were determined by ^{1}H-NMR.

Supplementary Figure S10. A Partial least squares discriminant analysis (PLS-DA) of metabolites from overexpression lines by ^{1}H-NMR.

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

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

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

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

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

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Figure legends:

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 0kg N/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 μm except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 μm. 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 four 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}H-NMR.

(a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null SE-3N by ^{1}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 (TGW) 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. NPTC: nucellar projection transfer cells. ETC: endosperm transfer cells. TaAAP2 expression localization 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 localization. AL: aleurone cells for TaAAP21 expression localization. ES: epithelium of scutellum for TaAAP21 expression localization. SC: scutellum. EM: embryo. : TaAAP2. :TaAAP13. : TaAAP21. : protein bodies

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Wheat amino acid transporters highly expressed in grain cells regulate grain size 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.

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 (AAAP), 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), ^{1}H-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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Introduction

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

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

Only a few AAT genes have been functionally characterized, mainly in Arabidopsis. Nevertheless, studies indicate that they play important roles in amino acid uptake into roots, phloem loading, long-distance transport and loading into the seed (Dinkeloo *et al.*, 2017; Tegeder, 2012). Crucial functions for the import of amino acids into sink tissues (seed, fruit, and tuber) have been described for different amino acid transporters. The AtAAP1 transporter is highly expressed in the embryo epithelium (transfer cells) of Arabidopsis and involved in uptake and transport of amino acids from the endosperm into the embryo, with *ataap*1 mutants having lower seed protein content and seed weight (Sanders *et al.*, 2009). RNAi-mediated down-regulation of the StAAP1 transporter can reduce the levels of free amino acids by 50% in potato tubers (Koch *et al.*, 2003). The tonoplast-localized UMAMIT24 of Arabidopsis is able to transport the amino acids temporarily stored in the vacuoles of chalaza cells before being delivered to filial tissue, while the plasma membrane-localized UMAMIT 25 is expressed in the endosperm and pericarp and could mediate amino acid export from endosperm (Besnard et al., 2018). The UMAMIT18 transporter is present in vascular tissues and developing seeds, supporting accumulation nitrogen in developing siliques (Ladwig *et al.*, 2012). UMAMIT11 and 14 AATs are expressed in the chalaza (unloading domain) of developing seeds and UMANIT28 and 29 transporters export amino acids from the endosperm and pericarp, respectively. Single loss-of-function mutants of these four transporters resulted in the accumulation of high levels of free amino acids in the seeds and greatly reduced seed size (Müller *et al.*, 2015). By contrast, overexpression of VfAAP1 (which is highly expressed in embryonic storage parenchyma cells at early maturation) in both pea and

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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. ¶

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Vicia narbonensis (Narbon bean) seeds resulted in increased seed protein content (by 10-25%) and seed size (by 20-30%), by increasing import of amino acids into the embryo (Rolletschek *et al.*, 2005). Similarly, the simultaneous overexpression of PsAAP1 in the phloem and embryos of pea plants increased seed yield and seed storage protein levels when the plants were grown with highly abundant N, due to increased source-to-sink allocation of amino acids (Zhang *et al.*, 2015) and increased nitrogen use efficiency (Perchlik and Tegeder, 2017). The OsAAP6 transporter, which is highly expressed in the endosperm of rice, functions as a positive regulator of GPC and overexpression in rice greatly increased GPC in rice grain when overexpressed (Peng *et al.*, 2014). Tomato SlCAT9 is a tonoplast Glu/Asp/GABA transporter, and overexpression of SlCAT9 greatly affects the flavor profile of the tomato fruit by increasing the accumulation of these amino acids during fruit development (Snowden *et al.*, 2015).

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

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

Results

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

Previously three TaAAP genes (TaAAP2, TaAAP13, and TaAAP21) out of 100 homoeologous groups of amino acid transporters (283 genes) were shown to be highly expressed in the endosperm transfer cells, starchy endosperm cells and aleurone cells of wheat grains, respectively (Wan *et al.*, 2017). These genes were therefore selected to determine their gene expression patterns, spatial tissue localization and potential 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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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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The inner starchy endosperm cells did not exhibit any GUS staining even after overnight incubation. No expression was observed in the aleurone cells (Figure 2, Supplementary Figure S2a), demonstrating that TaAAP13 was specific for the starchy endosperm, and suggesting that the high expression level in the aleurone cells shown by RNA-seq resulted from contamination during tissue preparation (Pfeifer *et al.*, 2014). Similarly, no GUS staining was observed in the embryo of TaAAP13 promoter::GUS transgenic lines. The TaAAP13 GUS expression patterns imply that it may function in transporting amino acid across the starchy endosperm.

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

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

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

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

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

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

Overexpression of TaAAP13 increases grain nitrogen content and grain size

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

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

The grains were longer and wider, but more wrinkled in the overexpression lines with higher transgene copy numbers and higher expression of TaAAP13 (Figure 5f). To determine if the shriveled grains resulted from reduced accumulation of starch, three overexpression lines (P26-OE, P23-OE, and P24-OE) with significantly increased nitrogen concentrations in grains were selected and their total starch contents compared with P15-null and non-transgenic lines (P29-WT) (Figure 5e). The results showed that overexpression of TaAAP13 resulted in lower concentrations of total starch by 6.6-14.3% and by 9.8-17.3% compared to the null and non-transgenic lines, respectively, but did not 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}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 endopserm (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

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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 TaAAAP13 on ω-gliadins is consistent with the ω-gliadins being highly responsive to the nitrogen status (Wan *et al.*, 2014).

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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 determinining 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

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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 ...

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

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

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

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

Real time PCR. Real time PCR was performed using an ABI7500 (Applied Biosystems) thermocycler. cDNA diluted 1:5 was used for RT–qPCR in a 25 µl reaction with SYBR ® Green JumpStart ™ Taq ReadyMix (Sigma-Aldrich).

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

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

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

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

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

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

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

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

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

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

Grain area, length, width, moisture and biomass measurement. Seed parameters of area (size), length and width were measured using MARVIN- Digital Seed Analyzer SN 176 (Marvitech - Germany). Four biological replicates from 16 plants (200-400 grains from each biological replicate) were analyzed. Seed moisture was detected using Bruker Minispec mg20 TD-NMR Contrast Agent analyzer (Germany) with bespoke robot and recording software (ROHASYS, Dutch Robotics) by loading 7-10g seeds. Thousand grain weight (TGW) was converted into 15% seed moisture grain weight. Biomass (above ground stems and leaves) were oven dried at 80°C overnight.

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

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

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

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

paraformaldehyde in 0.1 M Sorenson's phosphate buffer ($NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$, pH 7.0) with 2.5 % (w/v) glutaraldehyde overnight. After dehydration in increasing concentrations of ethanol, the sections were embedded in LR White Resin for two weeks at room temperature and polymerized at 55°C. A Reichert-Jung Ultracut ultramicrotome was used to section the resin-embedded grains at 0.5 µm or 1 µm thickness for protein staining. Protein bodies were stained with 1 % (w/v) Naphthol Blue Black in 7 % (w/v) acetic acid. The slides were visualized under a Zeiss Axiophot microscope and images were acquired with a RetigaExi CCD digital camera (Qimaging, Surrey, BC, Canada) under bright field optics and MetaMorph software version 7.5.5 9 (Molecular Devices, Sunnyvale, CA, USA).

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

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

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Conflict of interests

The authors declare that they have no competing interests.

Author contributions

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

Supporting information:

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

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

Supplementary Figure S3. Complementation of yeast mutants by TaAAP2, TaAAP13, and TaAAP21.

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

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

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

Supplementary Figure S7. phenotypes of overexpression of TaAAP13 lines.

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

Supplementary Figure S9: Metabolites of wholegrain flour from overexpression lines were determined by ^{1}H-NMR.

Supplementary Figure S10. A Partial least squares discriminant analysis (PLS-DA) of metabolites from overexpression lines by ^{1}H-NMR.

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

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

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

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

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

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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 0kg N/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 µm except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 µm. ETC: endosperm transfer cells, EM: embryo, AL: aleurone cells, ES: epithelium of scutellum, SE: starchy endosperm, RP: root primordia.

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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 four 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}H-NMR.

(a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null SE-3N by ^{1}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 (TGW) 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. NPTC: nucellar projection transfer cells. ETC: endosperm transfer cells. TaAAP2 expression localization 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 localization. AL: aleurone cells for TaAAP21 expression localization. ES: epithelium of scutellum for TaAAP21 expression localization. SC: scutellum. EM: embryo. : TaAAP2. : TaAAP13. : TaAAP21. : protein bodies

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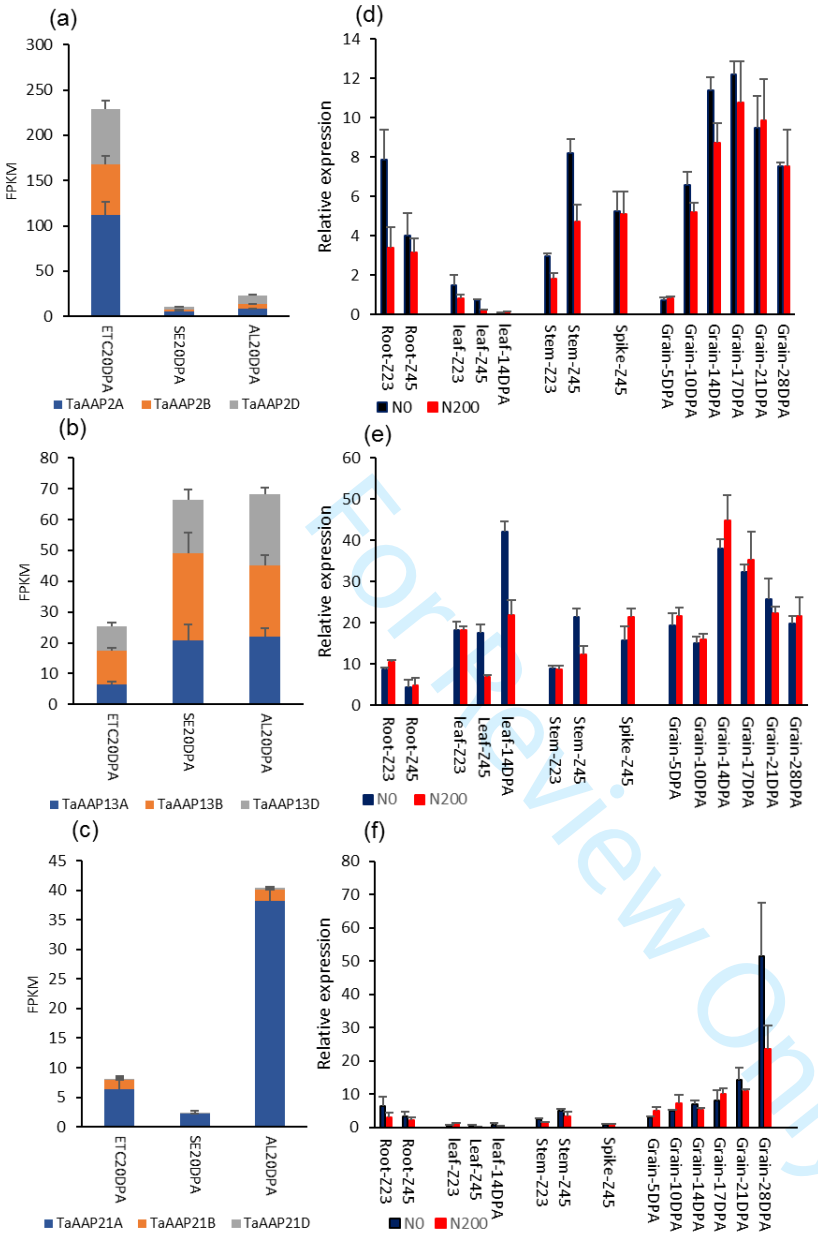

Figure 1: The expression patterns of TaAAP2, TaAAP13, and TaAAP21.

(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).

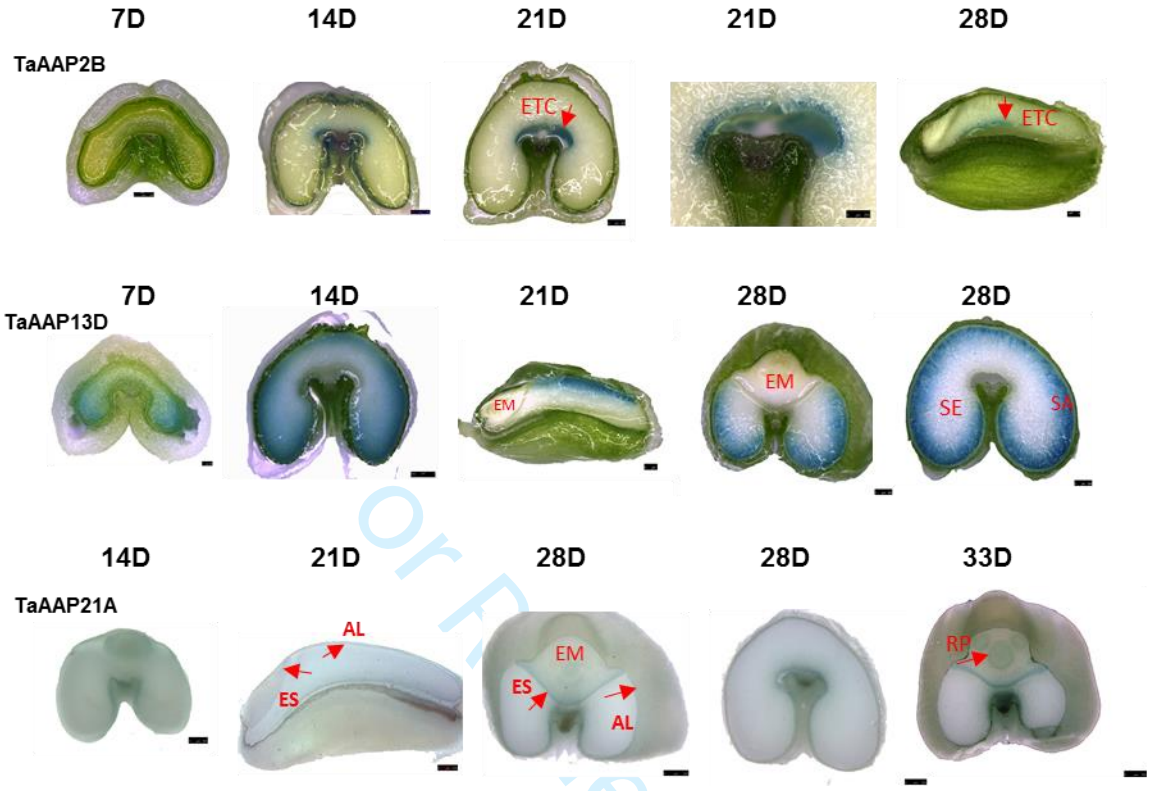

Figure 2. Localization of TaAAP2B, TaAAP13D, and TaAAP21A by promoter::GUS expression.

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 µm except TaAAP2B (21D enlarged) and TaAAP13D (7D) for 250 µm. ETC: endosperm transfer cells, EM: embryo, AL: aleurone cells, ES: epithelium of scutellum, SE: starchy endosperm, RP: root primordia.

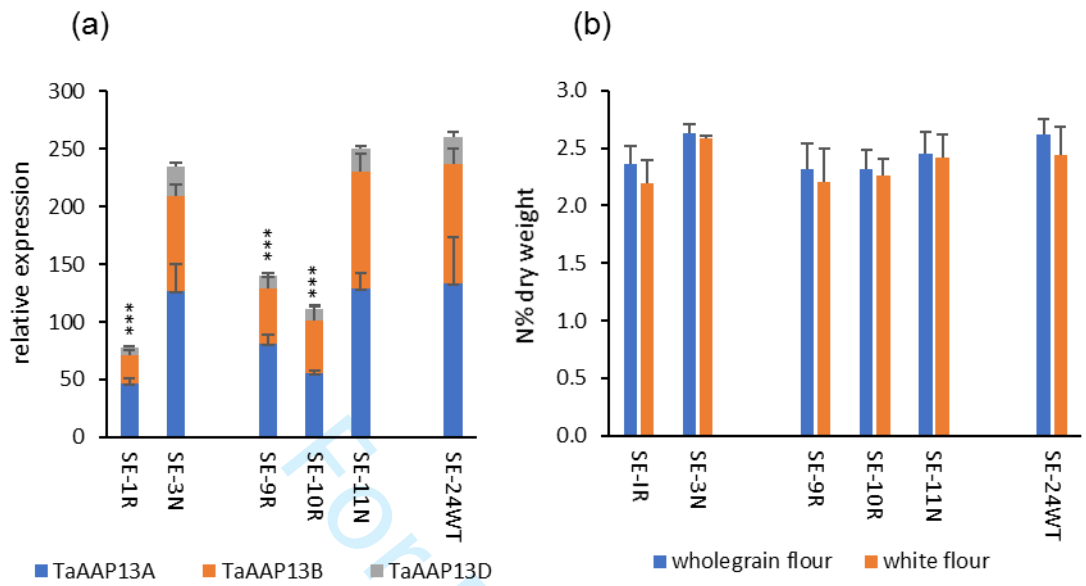

Figure 3. TaAAP13 expression and nitrogen concentrations in RNAi lines (SE-1R, SE-9R, SE-10R), null lines (SE-3N, SE-11N), and non-transgenic line SE-24WT (Cadenza, wild type).

(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 four and white flour. The differences between RNAi lines and nulls were not statistically significant (P<0.05, F-test).

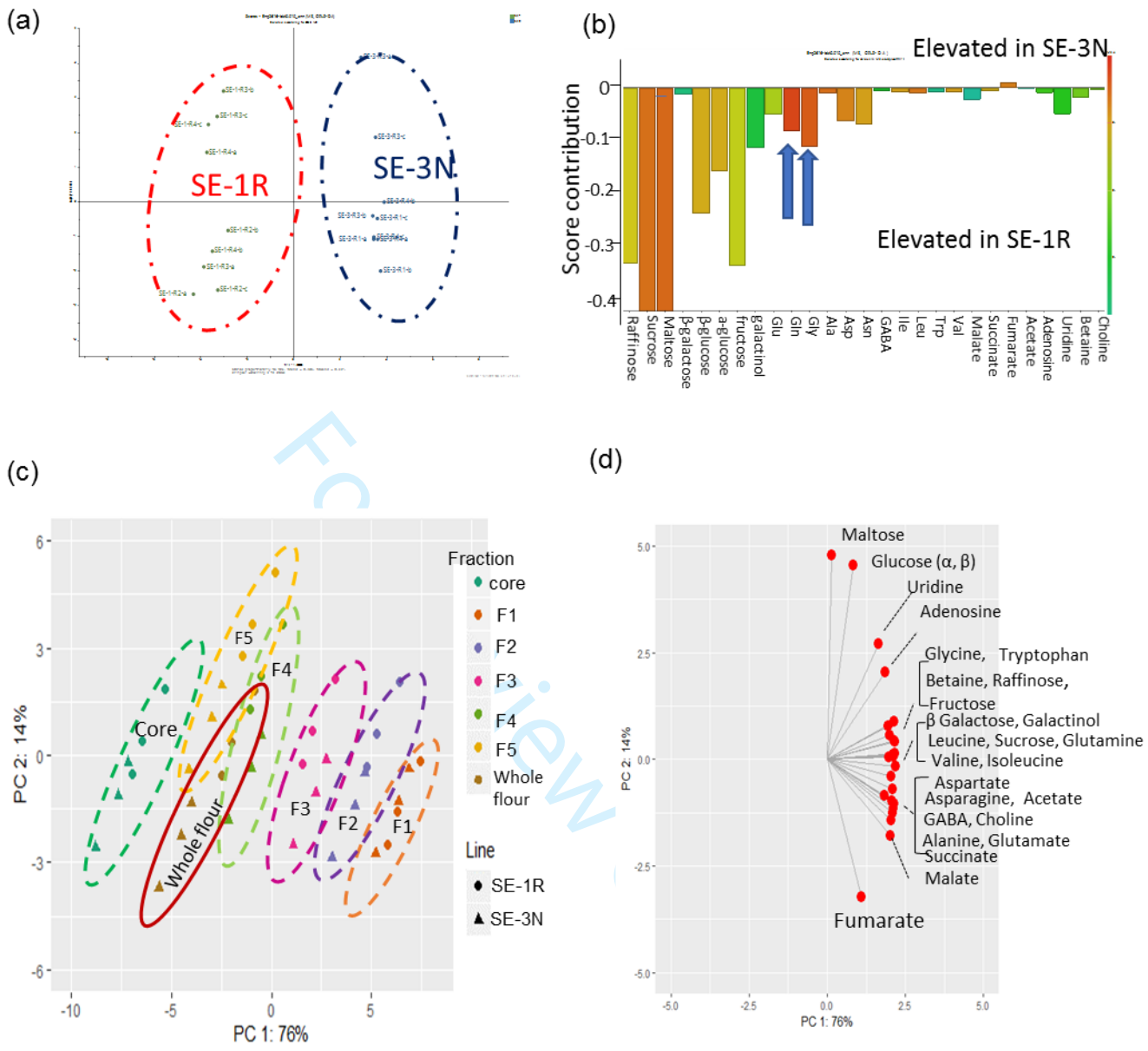

Figure 4. Metabolite data analysis of RNAi and null lines by ^{1}H-NMR.

(a). OPLS-DA of polar metabolite profiles of wholegrain flour in RNAi line SE-1R and null SE-3N by ^{1}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).

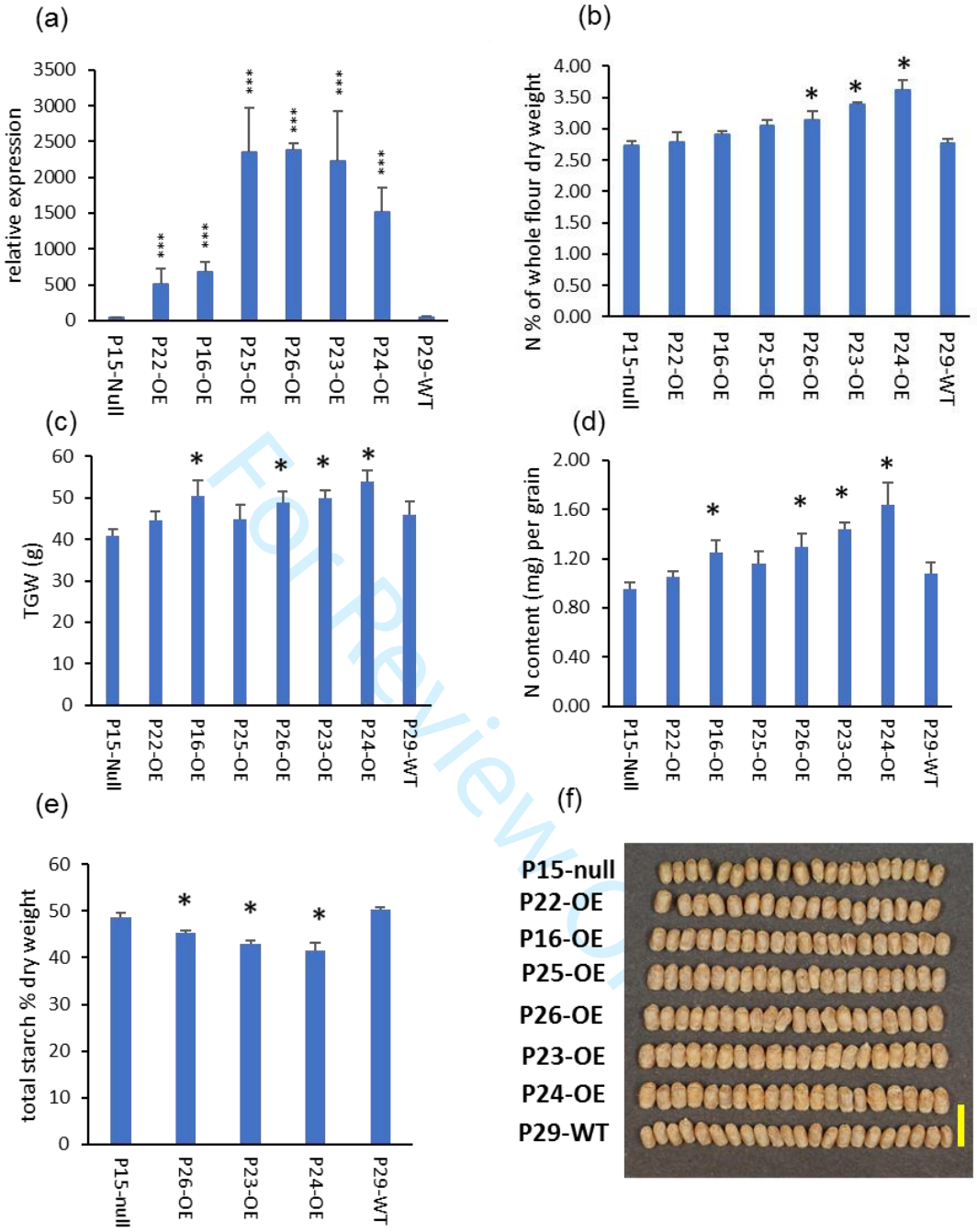

Figure 5. The gene expression and phenotype of overexpression TaAAP13 under the control of endosperm–specific promoter HMW-GS Dx5.(a). Gene expression of TaAAP13 at 21DPA in developing grains of overexpression lines: P22-OE and P16-OE (6 copies), P25-OE (16 copies), P26-OE (14copies), P23-OE (26 copies), 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 (***).

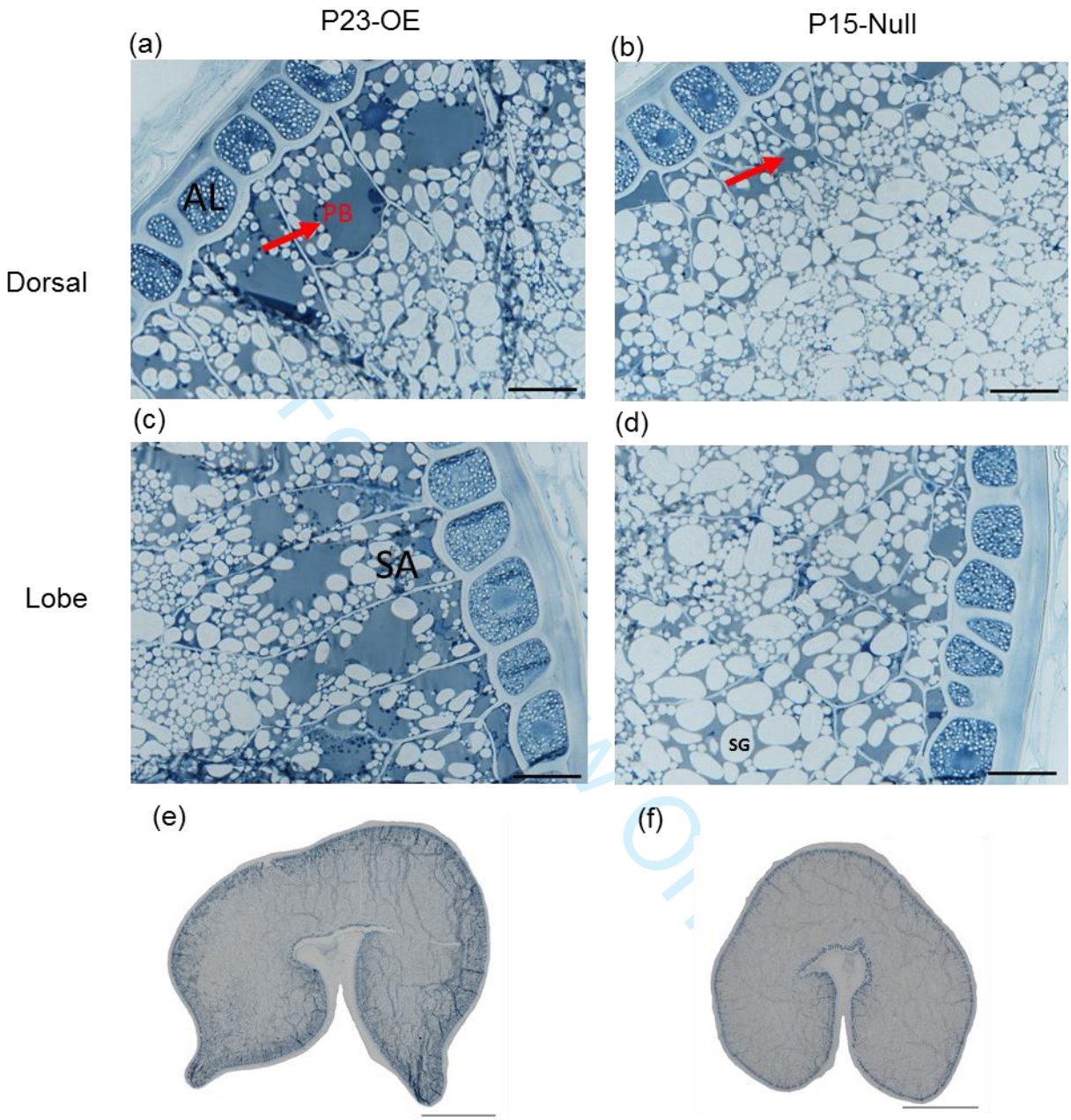

Figure 6. The protein distribution of mature grain resin sections of TaAAP13 overexpression line.

(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.

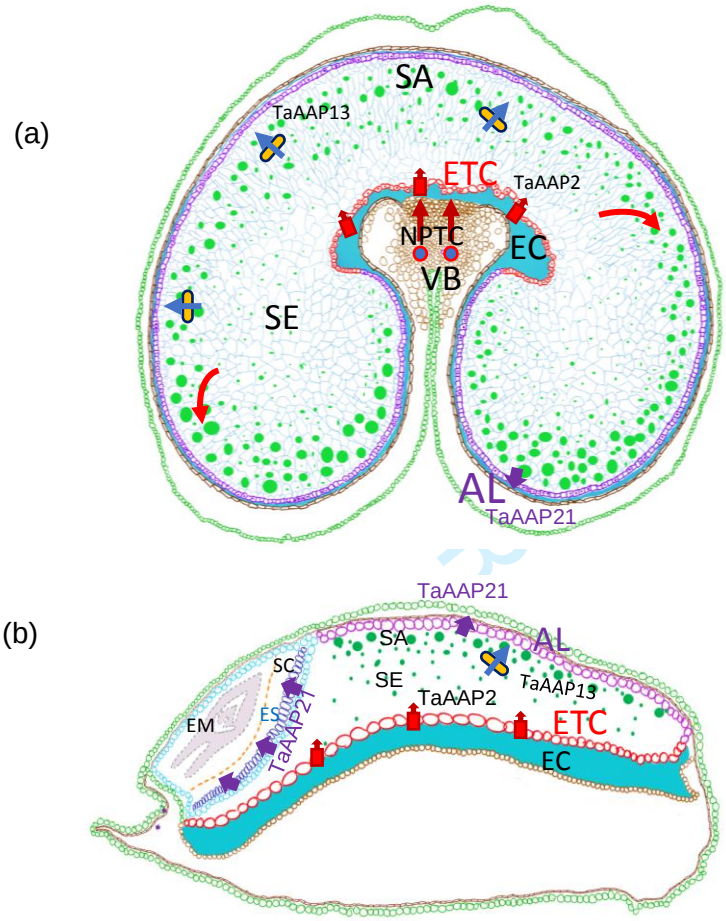

Figure 7. Schematic view of three TaAAPs nitrogen transport. (a). Wheat grain transverse section. (b) wheat grain longitudinal section. VB: vascular bundle. EC: endosperm cavity for amino acids delivered to endosperm from vascular bundle. NPTC: nucellar projection transfer cells. ETC: endosperm transfer 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.