Blockng amino acid transporter OsAAP3 improves grain yield by promoting outgrowth buds and increasing tiller number in rice

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Summary
Amino acid transporters (AATs) play indispensable roles in nutrient allocation during plant development. In this study, we demonstrated that inhibiting expression of the rice amino acid transporter OsAAP3 increased grain yield due to a formation of larger numbers of tillers as a result of increased bud outgrowth. Elevated expression of OsAAP3 in transgenic plants resulted in significantly higher amino acid concentrations of Lys, Arg, His, Asp, Ala, Gin, Gly, Thr and Tyr, and inhibited bud outgrowth and rice tillering. However, RNAi of OsAAP3 decreased significantly Arg, Lys, Asp and Thr concentrations to a small extent, and thus promoted bud outgrowth, increased significantly tiller numbers and effective panicle numbers per plant, and further enhanced significantly grain yield and nitrogen use efficiency (NUE). The promoter sequences of OsAAP3 showed some divergence between Japonica and Indica rice, and expression of the gene was higher in Japonica, which produced fewer tillers than Indica. We generated knockout lines of OsAAP3 on Japonica Zh11 and KY131 using CRISPR technology and found that grain yield could be increased significantly. These results suggest that manipulation of OsAAP3 expression could be used to increase grain yield in rice.

Introduction
Inorganic nitrogen (N) is mainly absorbed by plants in the form of nitrate and ammonium and is then converted into amino acids directly in the roots or after translocation to the leaves. The amino acids are then transported to roots, leaves, flowers, pollen and embryos (Fischer et al., 1998). Amino acids require transporter proteins to move them from source to sink organs (Coruzzi and Bush, 2001; Tegeder, 2012); these amino acid transporters (AATs) are cellular membrane proteins that transport particular amino acids. The transporters play critical roles in various processes in plants such as seed development, and abiotic and pathogen stresses (Näsholm et al., 2009; Paungfoo-Lonhienne et al., 2008; Schulze et al., 1999).

To date, a large number of AAT gene family members have been identified in Arabidopsis (Tegeder, 2012), rice (Lu et al., 2012), poplar (Wu et al., 2015), Solanum tuberosum L. (Ma et al., 2016) and Glycine max L. (Cheng et al., 2016). These studies have shown that AAP transporters play important roles in the loading of amino acids for nitrogen sink and supply (Tegeder and Ward, 2012). In Arabidopsis, AtAAP1 regulates amino acid transport to root cells and embryos (Lee et al., 2007; Sanders et al., 2009). In addition, AtAAP1 participates in the uptake of glutamate and neutral amino acids in Arabidopsis when these are present at soil concentrations (Perchl et al., 2014). AtAAP2 is localized in the phloem and plays a major role in N transfer from the xylem to phloem (Zhang et al., 2010). AtAAP3 is preferentially expressed in the root phloem (Okumoto et al., 2004). AtAAP5 transports amino acids at low concentrations in the roots (Svenerstam et al., 2008), and AtAAP6 regulates the amino acid composition of the phloem (Hunt et al., 2010). AtAAP8 transports amino acids to the endosperm during early embryogenesis (Schmidt et al., 2007) and was recently shown to be localized to the plasma membrane and to function in phloem loading (Santiago and Tegeder, 2016).

Although the functions of AtAATs have been extensively studied in Arabidopsis, the roles of OsAATs in rice are much less well understood (Zhao et al., 2012). Whole genome analyses have suggested the presence of 79–85 AAT homologous genes in rice (Lu et al., 2012; Zhao et al., 2012). It has been shown that biomass and yield of rice are altered significantly when OsAAT genes are knocked out (Lu et al., 2012; Peng et al., 2014). At present, it is known that OsAAP6 regulates grain protein content and nutritional quality in rice (Peng et al., 2014).

OsAAP3 has activity of transporting Ser, Met, Lys, Leu, His, Gln, Arg, Ala and Gly, especially for transporting the basic amino acids Lys and Arg (Taylor et al., 2015). Here, we further indicated that the SNPs in the promoter sequence of OsAAP3 are divergent between Japonica and Indica in all 524 rice accession varieties. And the expression level of OsAAP3 is negatively correlated with tiller number in rice. Blocking OsAAP3 could improve grain yield by promoting outgrowth buds and increasing tiller numbers especially in rice Japonica through regulating the concentrations of Lys, Arg, His, Asp, Ala, Gin, Gly, Thr and Tyr.

Results
Sequence divergence in the OsAAP3 promoter regions of Japonica and Indica rice

Overall, 524 rice accession varieties were used in this study, and these belong to nine subpopulations: IndI, IndII, Indica intermediate, Tej, Trj, Japonica intermediate, Aus, VI and
OsAAP3 regulates grain yield

intermediate (Chen et al., 2014). The Indica subpopulation (Indi, IndII and Indica intermediate) included 295 accessions, while the Japonica subpopulation (Tej, Trj and Japonica intermediate) included 156 accessions. We examined the exons and promoter sequences of OsAAP3 in all 524 accessions and identified 25 haplotypes in the accessions (Figure 1a). The Hap1 and Hap2 haplotypes were mainly present in Japonica accessions; the Hap4 haplotype was mainly found in Indica rice (Figure 1a). The Hapl2 and Hap4 haplotypes belonged to two separate evolutionary branches (Figure 1b). An average tiller number of 11 was found in accessions with the Hapl2 haplotypes, but was 17.72 in accessions with a Hap4 haplotype (Figure 1b).

OsAAP3 was mainly expressed in root, leaf, leaf sheath, culm and panicle (Figure 1c), especially in the root elongation area for lateral root growth, the basal part of the culm for bud outgrowth, the young leaf and the young panicle (Figure 1d). OsAAP3 showed higher expression level in the culm of Japonica accessions that carried the Hapl2 haplotypes compared with Indica accessions with the Hap4 haplotype (Figure 1e). We also found that the expression level of OsAAP3 was negatively correlated with tiller number (Figure 1f–g). The Japonica accessions had lower tiller numbers (Figure 1f), which was associated with the higher level of OsAAP3 expression in the culm (Figure 1g).

Down-regulation of OsAAP3 expression boosts grain yield by increasing tiller numbers in rice

To analyse the function of OsAAP3 in rice plants, we generated OE (overexpression) and Ri (RNA interference) transgenic lines (Figure 2c). To determine the effects of altered transcripts, whereas three Ri lines had low levels of that three OE lines had relatively high levels of lines (Ri1, Ri2 and Ri3) were significantly higher than that in ZH11 plants (Figure 2a, d). However, tiller numbers in OE3) were comparatively lower than that in wild-type ZH11 plants (Figure 2a, d). Root numbers (Figure S1a–c, e) and plant height (Figure S1a–c, f) also increased significantly under 1.0–8.0 mM nitrogen concentrations in Ri lines (Figure S1a–d). Root numbers (Figure S1a–c, e) and plant height (Figure S1a–c, f) also increased significantly under 1.0–8.0 mM nitrogen in Ri lines. These results indicated that RNAi-mediated down-regulation of OsAAP3 promoted bud outgrowth and seedling growth and might trigger the formation of an increased tiller number.

Effect of OsAAP3 expression on amino acid composition in rice straw and grain

As OsAAP3 is an amino acid transporter, we tested the effects of altering OsAAP3 expression levels on amino acid composition in rice straw and grain using the ninhydrin and HPLC method (Figure 4). In seedlings, total free amino acid concentrations in OE lines were increased significantly in roots and leaf sheaths, but decreased significantly in leaves (Figure 4a). By contrast, total free amino acid concentrations in Ri lines were decreased significantly in roots and leaf sheaths but increased significantly in leaves (Figure 4a). In addition, the total amount of amino acids in each tissue of OE line plants did not exceed that of ZH11 plants (Figure 4b), although the amino acid content in each tissue of Ri line plants was much higher than that in ZH11 plants (Figure 4b). To determine why amino acid concentrations were higher in OE lines, but overall amino acid content was lower, we measured the levels of individual amino acids in straw from OE and Ri lines. We found that the concentrations of Asp, Thr, Ser, Gly, Ala, Val, Ile, Leu, Tyr, Phe, Lys, Gln, His and Arg were increased significantly in OE lines (Figure 4c). However, the concentrations of Asp, Thr, Ser, Ile, Leu, Lys and Arg were decreased significantly in Ri lines (Figure 4c). A similar analysis of grains showed that the concentrations of Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, Leu, Tyr, Phe, Lys and Arg were increased significantly in OE lines (Figure 4d). However, none of the measured amino acids decreased in concentrations in grains of Ri lines (Figure 4d). These results showed that overexpression of OsAAP3 could increase the concentrations of amino acids, which inhibited the growth of plants; however, a reduction in expression of OsAAP3 prevented any inhibition of growth but did not affect the nutritional quality of the grain.

Increased concentrations of partial amino acids inhibit bud elongation and rice growth

As shown above, elevated expression of OsAAP3 caused an accumulation of amino acids, especially of Lys and Arg (Figure 4c), which can be directly transported by OsAAP3 (Taylor et al., 2015). However, bud elongation and plant growth were reduced in plants with elevated amino acids levels. Interestingly, our results showed that expression of OsAAP3 could be induced by exogenous Lys and Arg as its expression level peaked in roots after an 8-h treatment.
SNP divergence in the promoter sequence of OsAAP3 has SNP divergence between rice Japonica and Indica, and its expression level is associated with rice tiller. (a) SNP divergence in the promoter sequence of OsAAP3. (b) The phylogeny of promoter sequence of OsAAP3. (c) The expression level of OsAAP3 in different tissues in ZH11. (d) The root, basal part, leaf sheath, young leaf, old leaf, young panicle and old panicle GUS staining using pOsAAP3::GUS transgenic plant. (e) The average expression level of OsAAP3 in the culm of rice Japonica and Indica in a diverse worldwide collection of 524 O. sativa landraces (Chen et al., 2014). (f) Tiller number in ten cultivars Japonica and ten cultivars Indica. (g) The expression of OsAAP3 in the culm of ten cultivars Japonica and ten cultivars Indica.

(Figure S2a) and after 12 h in the basal part of the leaf sheath (Figure S2b). To understand the effects of these amino acids on plant growth and development, we added different amino acids to the nutrient solution in which wild-type ZH11 plants were growing. We found that low concentrations of Lys (0–1.0 mM) could promote the elongation of buds in rice (Figure S5, a, e), but high concentrations (1.0–2.0 mM) inhibited elongation of buds, especially for second buds (Figure S5, e). In the transgenic lines, bud elongation was inhibited in OE lines (Figure S5, b, f), but was accelerated in Ri lines (Figure S5, b, f), suggesting that the inhibitory effect was removed. In a similar fashion, high concentrations of Arg (0.2–0.4 mM) inhibited bud elongation (Figure S5, c, g), whereas low concentrations (0–0.2 mM) increased bud elongation (Figure S5, c, g). The inhibitory effect on bud growth could be prevented in Ri lines by treatment with 0.3 mM Arg (Figure S5d, h), although the effect was not as strong as seen for Lys treatment. We also indicated that very low concentrations (0.05–0.5 mM) of Asp, Ser, Gly and Tyr (Figure S3), low concentrations (0–1.0 mM) of Thr, Ala, Val, Leu and Gln (Figure S4) and medium concentrations (0.2–2.0 mM) of Ile, Phe and His (Figure S5) could promote the elongation of buds in rice, but enhanced concentrations of those amino acids inhibited the elongation of buds, especially for second buds (Figures S3-S5). In the transgenic lines, bud elongation was inhibited in OE lines, but was accelerated in Ri lines with the treatments of Asp, Gly, Tyr (Figure S3), Thr, Ala, Gln (Figure S4) and His (Figure S5). Bud elongation of all transgenic lines was accelerated under the treatments of Ile, Phe and Leu, but was inhibited under the treatments of Ser and Val, suggesting that the transgenic plants of OsAAP3 have no physiological effects under amino acids Ile, Phe, Leu, Ser and Val.

Root length, root number and plant height were decreased significantly by a 1.5 mM Lys treatment in OE lines (Figure S6a, c-e); however, all three traits increased in similarly treated Ri lines, especially root number and plant height (Figure S6a, c-e). Root length, root number and plant height were decreased significantly by a 0.3 mM Arg treatment in OE lines (Figure S6b, f-h), but were increased significantly in Ri lines (Figure S6b, f-h). The results described above indicate that Lys and Arg are not only amino acids absorbed by plants but also play important roles in regulating the growth and development of plants. Shoot branching (tiller) and growth are regulated by plant hormones, particularly cytokinins (CKs) (Ferguson and Beveridge, 2009). Levels of cytokinins were regulated through the irreversible oxidative cleavage of the N6-side chain by CYTOKININ DEHYDROGENASE/OXIDASE (CKXs) (Zurcher and Muller, 2016). To investigate the possible interaction with CKs, we measured the gene expression of 11 CKXs and found most of OsCKX5 (OsCKX2, OsCKX3, OsCKX4, OsCKX5, OsCKX6, OsCKX8, OsCKX9 and OsCKX10) exhibited higher expression level in OE lines compared to that in ZH11, whereas most of OsCKX6 (OsCKX2, OsCKX3, OsCKX5, OsCKX6, OsCKX8, OsCKX9, OsCKX10 and OsCKX11) demonstrated lower expression level in both Ri lines and mutant than that in ZH11 (Figure S7). These results suggest that altered expression of OsAAP3 controlled axillary bud outgrowth possibly by regulating CK pathway in the axillary bud.

Down-regulation of OsAAP3 expression enhances nitrogen use efficiency

To investigate the role of OsAAP3 in nitrogen use efficiency (NUE), total N concentration was measured. Overexpression of OsAAP3 led to a higher total N concentration, whereas total N concentration was unchanged after down-regulation of OsAAP3 expression (Figure 6a). Total N content per plant in OE lines was lower than that in ZH11, but higher than in Ri lines (Figure 6b). To confirm that OsAAP3 participates in N transportation to the panicle, the total N levels of mature seeds in the transgenic rice lines were measured (Figure 6c, d). Total N concentration in mature seeds was increased in OE lines, but no significant difference was found between Ri lines and wild-type ZH11 (Figure 6c). Thus, total N content of rice seeds in OE lines was decreased, but increased significantly in Ri lines (Figure 6d). The straw dry weight per plant of OE lines was significantly lower than that of ZH11 plants (Figure 6e). By contrast, down-regulation of OsAAP3 expression increased significantly straw dry weight per plant (Figure 6e). The NUE of OE lines was decreased significantly relative to ZH11 plants, but was increased significantly in Ri lines (Figure 6f).

Down-regulation of OsAAP3 by CRISPR technology increases grain yield in both Japonica ZH11 and KY131

Kongyu 131 (KY131) is a Japonica variety with the largest planting area in China. We compared the expression level of OsAAP3 in Japonica ZH11 and KY131 and found a higher expression level in KY131 (Figure S8). We knocked out the OsAAP3 sequence in Japonica ZH11 and KY131 using CRISPR technology, as higher expression level of OsAAP3 limited rice tiller numbers. Targets 1 and 2 for knockout of the OsAAP3 gene with CRISPR technology are shown in Figure 7a, and sequencing results showing base deletions and insertions after OsAAP3-CRISPR in Japonica ZH11 and KY131 are shown in Figure 7b. The effects of OsAAP3 knockout on seedling growth and agronomic traits related to grain yield were measured in OsAAP3-CRISPR lines. The biomas of OsAAP3-CRISPR lines ZH11-C and KY131-C were greater than those of ZH11 or KY131 (Figure 7c–f). Tiller number (Figure 7g–h), biomass of straw (Figure 7i–j), grain yield (Figure 7j–k) and NUE (Figure 7l) were also increased significantly in OsAAP3-CRISPR lines both in ZH11 or KY131 background compared with each wild-type.

Based on these results, we propose a model in which OsAAP3 participates in elongating outgrowth bud and increasing rice tiller number to modify grain yield through regulation the concentrations of Lys, Arg, His, Asp, Ala, Gln, Gly, Thr and Tyr in rice (Figure 8). In OE lines or Japonica, elevated expression of OsAAP3 may accumulate the concentrations of these amino acids. Hence,
Figure 2 Phenotypes of the rice plants with altered expression of OsAAP3 grown in paddy field. Whole plant phenotype (a) and filled grain number per plant (b) of wild-type ZH11, OsAAP3 overexpressing lines (OE1-OE3) and OsAAP3-RNAi lines (Ri1-Ri3). The expression of OsAAP3 in leaf (c), tiller number per plant (d), filled grain number per plant (e) and grain yield per plant (f) ZH11, OE1-OE3 lines and Ri1-Ri3 lines. Scale bars, 15.0 cm in (a) and 5.0 cm in (b). Values are means ± SD (n > 30). Significant levels: ***P < 0.001; **P < 0.01; *P < 0.05.
axillary bud is inhibited, which is unfavourable for rice tillering. However, Indica or RNAi/CRISPR line of OsAAP3 induces the opposite effects.

Discussion

Reduced expression of OsAAP3 can increase significantly tiller numbers, but does not change the nutritional quality of rice grains

Rice tillering is an important agronomic trait as it determines panicle number and grain yield (Li et al., 2003a,b; Xing and Zhang, 2010). In our study, we found that tiller numbers increased significantly in Ri lines at the productive stage (Figure 2a, b, d) compared with wild-type ZH11 and were also elevated in OsAAP3-CRISPR lines compared with wild-type ZH11 or KY131 (Figure 7g, h). By contrast, tiller numbers were reduced significantly in OE lines (Figure 2a, b, d). Compared to wild-type, Ri lines had a higher filled grain number per plant (Figure 2b, e), whereas OE lines had a reduced filled grain numbers per plant (Figure 2b, e). Our results also indicated that reduced expression of OsAAP3 could enhance significantly grain yield per plant (Figure 2f). OsAAP3-CRISPR lines gave similar results as described above and showed an increased grain yield per plant (Figure 7j, k). Similarly, Arabidopsis ataap2 mutants increase branch and silique numbers per plant and seed yield are strongly increased (Zhang et al., 2010).

Rice nutritional quality is another important trait that is used in breeding of new rice varieties (Zhang, 2007). Rice grain quality includes eating and cooking quality, nutritional quality, milling quality and appearance quality (Li et al., 2003a,b). The rice amino acid transporter OsAAP6 regulates starch storage and also affects protein storage in rice endosperm (Peng et al., 2014). In our study, a reduction in the level of expression of OsAAP3 did not cause any change in amino acid concentrations in grains (Figure 4d). Additionally, total nitrogen concentration was unchanged compared with wild-type ZH11 (Figure 6d). Plant membrane transporter genes can be incorporated into programs to enhance crop yields (Schroeder et al., 2013). Therefore, our systematic study of the function of transporter OsAAP3 is important for future genetic improvements in rice to increase grain yield and nutritional quality.

Partial amino acid concentrations influence bud outgrowth and tiller number in rice through the amino acid transporter OsAAP3

Rice tillers are produced by shoot branching, which consists of two distinct steps: first, formation of an axillary bud at each leaf axil; and second, outgrowth of the axillary bud (Li et al., 2003a,b; Xing and Zhang, 2010). Therefore, final tiller number is determined not only by how many tiller buds are formed but also by how many tiller buds are capable of outgrowth (Wang and Li, 2011). Axillary bud outgrowth is regulated by environmental signals (Xing and Zhang, 2010). Nitrogen is a crucial determinant of plant growth and crop productivity (Hachiya and Sakakibara, 2017; Li et al., 2017). Plants make use of transporters to take up N from the soil via the roots and transport it to other organs. Our study indicated that low concentrations of amino acids could promote the elongation of axillary buds in rice (Figure 5; Figures S3-S5), and that high concentrations of amino acids could inhibit axillary bud elongation, especially for second buds (Figure 5; Figures S3-S5). OsAAP3 has activity of transporting Ser, Met, Lys,
Leu, His, Gln, Arg, Ala and Gly, especially for transporting the basic amino acids Lys and Arg (Taylor et al., 2015). In the present study, we found that elongation of buds was inhibited by higher concentrations of Lys, Arg, His, Asp, Ala, Gln, Gly, Thr and Tyr in both OE lines and ZH11 (Figure 5; Figures S3-S5), but was increased in Ri lines (Figure 5; Figures S3-S5). These results suggest that the inhibitory effect triggered by high concentrations of amino acids was ameliorated in lines with reduced expression of OsAAP3. Enhanced translocation of Lys, Arg, His, Ala, Gln and Gly to the basal parts of the axillary buds in OE lines might contribute to the inhibition of growth of axillary buds. Thus, an appropriate level of expression of the transporter gene OsAAP3 is required to support plant growth.

It has been reported that Lys can inhibit mitotic activity in the root apical meristem, and that exogenous Lys can reduce the length of the main root of Arabidopsis (Yang et al., 2014). Lys and Arg can be transported by the amino acid transporter AtAAP3 in Arabidopsis. The root system of AtAAP3 mutant is highly developed and shows a larger number of long main roots and a higher density of lateral root (Marella et al., 2013). OsGS1.2, which is a major gene for nitrogen assimilation, has been shown to promote axillary bud outgrowth and increase tiller numbers in rice (Obara et al., 2004; Ohashi et al., 2017). In contrast, bud outgrowth marker gene OsFC1 is required for axillary buds outgrowth and has been reported to inhibit rice tiller number (Minakuchi et al., 2010). In our study, expression of OsGS1.2 was down-regulated in OE lines and up-regulated in Ri lines (Figure 3d); however, expression of OsFC1 was up-regulated in OE lines and down-regulated in Ri lines (Figure 3e). Furthermore, it was reported that down-regulation of OsCKX2 expression increases tiller number and improves rice yield (Yeh et al., 2015). Our results showed that higher expression of OsCKXs in OE lines and lower expression of OsCKXs in Ri lines than that in ZH11 (Figure S7) indicated that altered expression of OsAAP3 controlled axillary bud outgrowth possibly by regulating CK pathway in the axillary bud.

Additionally, amino acids are used for basic metabolism and protein synthesis in the leaf, and then modulate plant growth (Yadav et al., 2015). Our results showed that total free amino acid concentrations in the leaves of OE lines were decreased significantly, but increased significantly in the leaves of Ri lines (Figure 4a). Starting with less outgrowth buds, less tiller numbers

Figure 4 Effect of OsAAP3 on amino acid content in ZH11, OsAAP3 overexpressing lines (OE1-OE2) and OsAAP3-RNAi lines (Ri1-Ri2). Total free amino acid concentration (a) and total free amino acid content per plant (b) in root, leaf sheath, leaf of ZH11, OE1-OE2 lines and Ri1-Ri2 lines at seedling stage. Amino acid concentration of straw at filling stage (c) and grain at mature stage (d) in ZH11, OE1-OE2 lines and Ri1-Ri2 lines grown in paddy. Values are means ± SD (n > 3). Significant levels: ***P < 0.001; **P < 0.01; *P < 0.05.
and poor plant growth of OE lines (Figure 2a) might result from lacking of amino acids. We further indicated that repression of OsAAP3 could promote bud outgrowth and seedling growth, and trigger the development of a larger number of tillers through regulating the allocation and utilization of amino acids by enhancing the ability of nitrogen balance.

Inhibition of OsAAP3 expression increases grain yield and NUE in Japonica rice varieties

Haplotype-level association analysis is an important tool for molecular plant breeding (Han et al., 2016). It has been reported that the NRT1.1B-indica allele can increase tiller numbers per plant, enhance grain yield per plant and increase yield per plot under high N level (Hu et al., 2015). In the present study, we analysed the promoter and coding regions of OsAAP3 in a diverse collection of 524 O. sativa landraces. Some differences were found in SNPs in the promoter sequences of OsAAP3 of Japonica (Hap1 and Hap2) and Indica (Hap4) accessions. Expression of OsAAP3 was higher in the Japonica accessions. Japonica rice has well-known nutritional qualities but produces fewer tillers and has a lower NUE than Indica rice (Hu et al., 2015; Koutroubas and Ntanos, 2003). Reduced expression of OsAAP3 can increase yield and NUE and does not affect nutritional quality. Therefore, inhibiting expression of OsAAP3 in Japonica rice may be important for improving tillering and grain yield. CRISPR technology is an effective method for inhibiting gene expression in plants (Ma et al., 2015; Yin et al., 2017). Our results indicated that tiller numbers and grain yield (Figure 7) increased significantly in OsAAP3-CRISPR lines on a Japonica ZH11 or KY131 background. Thus, inhibition of OsAAP3 expression may contribute to an improved grain yield and NUE for Japonica varieties.

Experimental procedures

Construction of OsAAP3 altered expression and promoter-GUS vector

To construct OsAAP3 overexpressing plants, a 1464 bp OsAAP3 cDNA containing the open reading frame (ORF) of OsAAP3 (LOC_Os06g36180) was inserted downstream of the 35S promoter in pCAM1306 using Kpn I and Xba I to produce the plasmid p35S-OsAAP3. To generate the OsAAP3-RNAi construct, two fragments of OsAAP3 cDNA (309 bp) were amplified by PCR using the primers listed in Table S1 and transferred downstream of the Ubi-1 promoter in the rice RNAi vector pTCK303 (Wang et al., 2004) using BamH I/Xba I, and Spe I/Sac I, respectively, to generate the OsAAP3-RNAi vector pOsAAP3-RNAi. To analyse the OsAAP3 promoter, a 2179-bp OsAAP3 promoter fragment was generated by PCR using the primers shown in Table S1, and inserted in front of the β-glucuronidase (GUS) coding region in pCAMBIA1391Z with Hind III and Nco I to generate the pOsAAP3-GUS plasmid.

Construction of OsAAP3 CRISPR vector

The OsAAP3 CRISPR vector construct was prepared using CRISPR/Cas9-based multiplex genome editing for monocot and dicot plants (Ma et al., 2015). Two target sequences of OsAAP3 were designed and added to U6IPAT/U6OPST or U3IPAT/U3OPST primers. A 422-bp fragment from target sequence 1 was amplified by PCR using U6OPST and AAP3-U6IPAT primers and the U6 plasmid. In the same way, a 348-bp fragment was amplified by PCR from target sequence 2 using ZRO589 and AAP3-U3IPAT primers with the U3 plasmid. A 476-bp fragment from target sequence 2 using ZRO589 and AAP3-U3IPAT primers was amplified by PCR from target sequence 2 using ZRO589 and AAP3-U3IPAT primers with the U3 plasmid. A 476-bp fragment from target sequence 2 using ZRO589 and AAP3-U3IPAT primers with the U3 plasmid.
sequence 1 was amplified by PCR using AAP3-U6OPST and U6IPAT primers with the U6 plasmid. Similarly, a 463-bp fragment from target sequence 2 was amplified by PCR using AAP3-U3IPST and ZRO282 primers with the U3 plasmid. Complete U6 and U3 fragments were amplified by fusion PCR containing U6 promoter-sgRNA and U3 promoter-sgRNA. The U6 fragment was digested with Kpn I, and the U3 fragment was digested with Kpn I and Sac I and then the U6 and U3 fragment were connected. The complete U6 and U3 sequences of 1600 bp were amplified by PCR using U6OPST and ZRO282 primers with the U6–U3 plasmid. Finally, the complete U6–U3 fragment of 1600 bp was inserted into the Per8-Cas9 vector using Kpn I and Sac I and cloning kits.

**Acquisition and detection of transgenic plants**

All of the above-mentioned constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 (Hiei et al., 1997). The Japonica rice variety Zhonghua 11 (ZH11) and Kongyu 131 (KY131) were transformed by *Agrobacterium*-mediated

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**Figure 6** Effect of OsAAP3 on nitrogen content in ZH11, OsAAP3 overexpressing lines (OE1-OE3) and OsAAP3-RNAi lines (Ri1-Ri3). (a) Total nitrogen concentrations of straw of ZH11, OE1-OE3 and Ri1-Ri3 line at filling stage grown in paddy. (b) Total nitrogen content per plant of ZH11, OE1-OE3 and Ri1-Ri3 lines at filling stage grown in paddy. (c) Total nitrogen concentrations of grain of ZH11, OE1-OE3 and Ri1-Ri3 lines at filling stage grown in paddy. (d) Total nitrogen content of grain of ZH11, OE1-OE3 and Ri1-Ri3 line at filling stage grown in paddy. (e) Straw dry weight of ZH11, OE1-OE3 and Ri1-Ri3 lines at mature stage grown in paddy. (f) Nitrogen use efficiency (NUE) of ZH11, OE1-OE3 and Ri1-Ri3 lines at mature stage grown in paddy. Values are means ± SD (n > 3). Significant levels: ***P < 0.001; **P < 0.01; *P < 0.05.
transformation with 50 mg/L hygromycin for selecting transgenic calli (Hiei et al., 1997). The T2 homologous transgenic lines (overexpression and RNAi) with altering expression were selected using PCR and the primers listed in Table S1. The T0 CRISPR transgenic lines were selected using PCR sequencing and the corresponding primers in Table S1.

Hydroponic culture and field experiments

Hydroponic experiments were conducted using basic rice culture solution (Yoshida et al., 1976) without N under natural rice growth conditions; the N content was adjusted in each experiment. To analyse the phenotype of OsAAP3 transgenic plants in the presence of different concentrations of NH4NO3, seedlings were cultivated in basic nutrient solution supplemented with 0.25 mM, 0.5 mM, 1.0 mM, 2.0 mM or 4.0 mM NH4NO3. To investigate the effect of each amino acid on the phenotype of OsAAP3 transgenic plants, seedlings were grown in basic nutrient solution with 1.0 mM NH4NO3 as the N source for 1 week, then transferred to basic nutrient solution supplemented with 1.0 mM NH4NO3 and each amino acid as the N source. A collection of 524

Figure 7 Blocking OsAAP3 improves grain yield by increasing tiller number in rice. (a) Target 1 and target 2 of gene knockout for OsAAP3 with CRISPR technology. (b) Sequencing results of base deletion and insertion of OsAAP3-CRISPR in Japonica ZH11 and KY131. On the right, minus (-) and plus (+) signs indicate the number of nucleotides deleted and inserted at OsAAP3-CRISPR target sequence site 1 and site 2, respectively. Phenotypes of OsAAP3 CRISPR lines (three) in ZH11 (c) and in KY131 (d). Biomass analyses of OsAAP3 CRISPR lines (three) in ZH11 (e) and in KY131 (f). Whole plant phenotype of OsAAP3 CRISPR line in ZH11 and in KY131 (g). Tiller number per plant (h), biomass of straw (i), grain yield per plant (j-k) and NUE (l) analyses of OsAAP3-CRISPR line in ZH11 and in KY131. Scale bars, 3 cm in (c), 3 cm in (d), 15 cm in (g) and 2.5 cm in (j). Values are means ± SD (n > 20). Significant levels: ***P < 0.001; **P < 0.01; * P < 0.05.
O. sativa landraces (Chen et al., 2014) was used in this study. Tiller numbers were counted at the filling stage over three seasons from 2014 to 2017. Field experiments were carried out in an experimental field at Wuhan Institute of Bioengineering, China, during the rice growing season.

**GUS staining and quantitative RT-PCR analysis**

Histochemical GUS staining was performed according to Jefferson et al. (1987). The stained tissues were rinsed and fixed in FAA (formalin-acetic acid-70% ethanol [1:1:18]) at 4 °C for 24 h. Then the stained materials were observed using stereo microscope. Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, http://www.invitrogen.com). First-strand cDNA was synthesised from 3 μg of total RNA from each sample using M-MLV reverse transcriptase (Promega, http://www.promega.com). Quantitative RT-PCR (qRT-PCR) was performed to monitor gene expression, and OsActin1 (LOC_Os03g50885) was used as a control. qRT-PCR was carried out in the presence of the double-strand DNA-specific dye SYBR Green (Takara, China) and monitored in real time with a 7500 qRT-PCR system (Applied Biosystems, Singapore).

**Nitrogen and amino acid analysis**

Total free amino acid concentrations were measured by the ninhydrin method (Fang et al., 2013). Single free amino acid concentrations were measured using HPLC with an amino acid analyser L-8800 HITACHI. The samples were prepared as follows: 1.0 g rice tissue was placed in 80% ethanol (10.0 mL) at 80 °C in a

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Figure 8: The proposed model of OsAAP3 regulating rice tillering and grain yield. In OE or Japonica lines, elevated expression of OsAAP3 accumulates basic amino acid (Lys, Arg and His), acidic amino acid (Asp), neutral amino acid (Ala, Gin, Gly, Thr and Tyr). Hence, axillary bud is inhibited, which is unfavourable for rice tillering. However, RNAi/CRISPR line or Indica of OsAAP3 induces the opposite effects. The red arrows mean first tiller bud and second tiller bud, the blue arrows mean the development of rice OE lines or Japonica (such as KY131) from seedling to mature plant, the green arrow means the development of rice wild type (such as Japonica ZH11) from seedling to mature plant, and the pink arrows mean the development of rice RNAi/CRISPR line or Indica from seedling to mature plant. The blue circles mean basic amino acid (Lys, Arg and His), the yellow circles mean acidic amino acid (Asp), and the pink circles mean neutral amino acid (Ala, Gin, Gly, Thr and Tyr).
water bath for 20 min; this step was repeated twice. The collected extracts were placed at 80 °C in a drying oven to remove the ethanol, and 1 mL 0.5 M NaOH was used to dissolve the sediment. The solution was centrifuged at 12000 g for 15 min. The supernatant was collected and filtered through a filter membrane (2 μm); 0.8 mL of each filtrate was analysed using the amino acid analyser. Total nitrogen content and total protein content were determined using the semimicro Kjeldahl method using a nitrogen analyser (Smart Chem 200, Westco, Italy). Nitrogen utilization efficiency was determined from the formula:

\[ \text{NUE}(\%) = \frac{\text{[yield of grain] (g)}}{\text{[nitrogen content of grain] (g)}} \times \frac{\text{straw nitrogen content (g)}}{\text{C138/C2}} \times 100. \]

Bud outgrowth analysis
Germinated seeds were transferred into a hydroponic culture box with sterile water and cultured at 28 °C and 60% relative humidity under white light with a 16-h light/8-h dark photoperiod for 3 days. They were then grown with rice culture solution for an additional 6 d under the same conditions. Then, seedlings for bud outgrowth analysis were placed in rice culture solution in a glasshouse at 32 °C under a high voltage sodium lamp (400 W) for 14 h, and at 25 °C in the dark for 10 h in the evening. The nutrient solution was renewed every 3 days. The length of bud outgrowth was measured using stereo microscope with ImageJ software.

SNP database
In total, 524 accessions were genotyped via sequencing (Chen et al., 2014). SNP information is available on RiceVarMap (http://ricevarmap.ncpgr.cn/), which is a comprehensive database of rice genomic variations. SNP physical locations were obtained from the TiGR Rice Loci 6 genome in RiceVarMap. Haplotypes with an allele frequency of 0.01 or higher (five accessions) were used for association analysis via ANOVA. Duncan’s multiple range test was applied to identify differences in SNP content between all possible haplotype pairs.

Statistical analysis
For all treatments, the statistical differences are indicated by asterisks, and the Student’s t-test allowing the determination of the significance between two sets of data (transgenic line vs wild-type ZH11 or KY131) is performed using the SPSS 10 software (IBM, Inc.). Significant levels: ***p < 0.001; **p < 0.01; *P < 0.05.

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References
Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S. and Kyoizuka, J. (2009) d14, a Strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. Plant Cell Physiol. 50, 1416–1424.

Chen, W., Gao, Y., Xie, W., Gong, L., Lu, K., Wang, W., Li, Y. et al. (2014) Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. Nat. Genet. 46, 714–721.

Cheng, L., Yuan, H.Y., Ren, R., Zhao, S.Q., Han, Y.P., Zhou, Q.Y., Ke, D.X. et al. (2016) Genome-wide identification, classification, and expression analysis of amino acid transporter gene family in Glycine Max. Front. Plant Sci. 7, 1–14.

Coruzzi, G. and Bush, D.R. (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiol. 125, 61–64.

Fang, Z., Xia, K., Yang, X., Grotemeyer, M.S., Meier, S., Rentsch, D., Xu, X. et al. (2013) Altered expression of the PTR/NTT homologue OsPTR9 affects nitrogen utilization efficiency, growth and grain yield in rice. Plant Biotechnol. J. 11, 446–458.

Ferguson, B.J. and Beveridge, C.A. (2009) Roles for auxins, cytokinins, and strigolactones in regulating shoot branching. Plant Physiol. 149, 1929–1944.

Fischer, W.N., Andere, B., Rentsch, D., Kroliekiewicz, S., Tegeder, M., Breitkreuz, K. and Frommer, W.B. (1998) Amino acid transport in plants. Trends Plant Sci. 3, 188–195.

Hachiya, T. and Sakakibara, H. (2017) Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. J. Exp. Bot. 68, 2501–2512.

Han, Z., Zhang, B., Zhao, H., Ayaad, M. and Xing, Y. (2016) Genome-wide association studies reveal that diverse heading date genes respond to short and long day Lengths between Indica and Japonica rice. Front. Plant Sci. 7, e1003281.

Hiei, Y., Komari, T. and Kubo, T. (1997) Transformation of rice mediated by Agrobacterium tumefaciens. Plant Mol. Biol. 35, 205–218.

Hu, B., Wang, W., Du, S., Tang, J., Li, H., Che, R., Zhang, Z. et al. (2015) Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. Nat. Genet. 47, 834–838.

Hunt, E., Galtotin, S., Newbury, H.J., Bale, J.S., Tseng, H.M., Barrett, D.A. and Pritchard, J. (2010) A mutation in amino acid permease AAP6 reduces the amino acid content of the Arabidopsis sieve elements but leaves aphid herbivores unaffected. J. Exp. Bot. 61, 55–64.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901.

Koutrobaus, S.D. and Ntanos, D.A. (2003) Genotypic differences for grain yield and nitrogen utilization in Indica and Japonica rice under Mediterranean conditions. Field Crops Res. 83, 251–260.

Lee, Y.H., Foster, J., Chen, J., Voll, L.M., Weber, A.P. and Tegeder, M. (2007) AAP1 transports uncharged amino acids into roots of Arabidopsis. Plant J. 50, 305–319.

Li, X., Qian, Q., Fu, Z., Wang, Y., Xiong, G., Zeng, D., Wang, X. et al. (2003a) Control of tillering in rice. Nature, 422, 618–621.

Li, Z., Wan, J., Xia, J. and Yano, M. (2003b) Mapping of quantitative trait loci controlling physico-chemical properties of rice grains (Oryza sativa L.). Jap. J. Breeding, 53, 209–215.

Li, H., Hu, B. and Chu, C. (2017) Nitrogen use efficiency in crops: lessons from Arabidopsis and rice. J. Exp. Bot. 68, 2477–2488 ext101.

Lu, Y., Song, Z., Lu, K., Lian, X. and Cai, H. (2012) Molecular characterization, expression and functional analysis of the amino acid transporter gene family (OsAAATs) in rice. Acta Physiol. Plant. 34, 1943–1962.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B. et al. (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol. Plant, 8, 1274–1284.

Ma, H.L., Cao, X.L., Shi, S.D., Li, S.L., Gao, J.P., Ma, Y.L., Zhao, Q. et al. (2016) Genome-wide survey and expression analysis of the amino acid transporter superfamily in potato (Solanum tuberosum L.). Plant Physiol. Bioch. 107, 164–177.

Marella, H.H., Nat, C., Nielsen, E., Schachtman, D.P. and Taylor, C.G. (2013) The amino acid permeases AAP3 and AAP5 are involved in root-knot nematode parasitism of Arabidopsis. Mol. Plant Microbe. In. 26, 44–54.

Minakuchi, K., Kameoka, H., Yasuno, N., Umehara, M., Luo, L., Kobayashi, K., Hanada, A. et al. (2010) FINE CULM1 (FC1) works downstream of strigolactones to inhibit the outgrowth of axillary buds in rice. Plant Cell Physiol. 51, 1127–1135.

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Näsholm, T., Kielland, K. and Ganeteg, U. (2009) Uptake of organic nitrogen by plants. New Phytol. 182, 31–48.

Obara, M., Sato, T., Sasaki, S., Kashiba, K., Nagano, A., Nakamura, I., Ebinti, T. et al. (2004) Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice. Theor. Appl. Genet. 110, 1–11.

Ohashi, M., Ishiyama, K., Kojima, S., Kojima, M., Sakakibara, H., Yamaya, T. and Hayakawa, T. (2017) Lack of cytosolic glutamine synthetase1;2 activity reduces nitrogen-dependent biosynthesis of cytokinin required for axillary bud outgrowth in rice seedlings. Plant Cell Physiol. 58, 679–690.

Okumoto, S., Koch, W., Tegeder, M., Fischer, W.N., Biehl, A., Leister, D., Stierhof, Y.D. et al. (2004) Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. J. Exp. Bot. 55, 2155–2168.

Paungfoo-Lonhienne, C., Lonhienne, T.G., Rentsch, D., Robinson, N., Christie, M., Webb, R.I., Gamage, H.K. et al. (2008) Plants can use protein as a nitrogen source without assistance from other organisms. Proc. Natl. Acad. Sci. USA, 105, 4524–4529.

Peng, B., Kong, H., Li, Y., Wang, L., Zhong, M., Sun, L., Gao, G. et al. (2014) OsAAP6 functions as an important regulator of grain protein content and nutritional quality in rice. Nat. Commun. 5, 4847.

Perchlik, M., Foster, J. and Tegeder, M. (2014) Different and overlapping functions of Arabidopsis LHT6 and AAP1 transporters in root amino acid uptake. J. Exp. Bot. 65, 5193–5204.

Sanders, A., Collier, R., Trethewy, A., Gould, G., Sieker, R. and Tegeder, M. (2009) AAP1 regulates import of amino acids into developing Arabidopsis embryos. Plant J. 59, 540–552.

Santiago, J.P. and Tegeder, M. (2016) Connecting source with sink: the role of Arabidopsis AAP8 in phloem loading of amino acids. Plant Physiol. 171, 508–521.

Schmidt, R., Stransky, H. and Koch, W. (2007) The amino acid permease AAP8 is important for early seed development in Arabidopsis thaliana. Planta, 226, 805–813.

Schroeder, J.J., Delhaize, E., Frommer, W.B., Guerinot, M.L., Harrison, M.J., Herrera-Estrella, L., Horie, T. et al. (2013) Using membrane transporters to improve crops for sustainable food production. Nature, 497, 60–66.

Schulze, W., Frommer, W.B. and Ward, J.M. (1999) Transporters for ammonium, amino acids and peptides are expressed in pitchers of the carnivorous plant Nepenthes. Plant J. 17, 637–646.

Svennerstam, H., Ganeteg, U. and Näsholm, T. (2008) Root uptake of cationic amino acids by Arabidopsis depends on functional expression of amino acid permease 5. New Phytol. 180, 620–630.

Taylor, M.R., Reinders, A. and Ward, J.M. (2015) Transport function of rice amino acid permeases (AAPs). Plant Cell Physiol. 56, 1355–1363.

Tegeder, M. (2012) Transporters for amino acids in plant cells: some functions and many unknowns. Curr. Opin. Plant Biol. 15, 315–321.

Tegeder, M. and Ward, J.M. (2012) Molecular evolution of plant AAP and LHT amino acid transporters. Front. Plant Sci. 3, 21.

Wang, Y. and Li, J. (2011) Branching in rice. Curr. Opin. Plant Biol. 14, 94–99.

Wang, Z., Chen, C., Xu, Y., Jiang, R., Han, Y., Xu, Z.H. and Chong, K. (2004) A practical vector for efficient knockdown of gene expression in rice (Oryza sativa L.). Plant Mol. Biol. Rep. 22, 409–417.

Wu, M., Wu, S., Chen, Z., Dong, Q., Yan, H.W. and Xiang, Y. (2015) Genome-wide survey and expression analysis of the amino acid transporter gene family in poplar tree. Genom. Genet. 11, 83.

Xing, Y. and Zhang, Q. (2010) Genetic and molecular bases of rice yield. Annu. Rev. Plant Biol. 61, 421–442.

Yadav, U.P., Ayre, B.G. and Bush, D.R. (2015) Transgenic approaches to altering carbon and nitrogen partitioning in whole plants: assessing the potential to improve crop yields and nutritional quality. Front. Plant Sci. 6, 275.

Yang, H., Postel, S., Kemmerling, B. and Ludewig, U. (2014) Altered growth and improved resistance of Arabidopsis, against pseudomonas Syringae by overexpression of the basic amino acid transporter ATCAT1. Plant Cell Envir. 37, 1404–1414.

Yeh, S.Y., Chen, H.W., Ng, C.Y., Lin, C.Y., Tseng, T.H., Li, W.H. and Ku, M.S. (2015) Down-regulation of cytokinin oxidase 2 expression increases tiller number and improves rice yield. Rice, 8, 36.

Yin, K.Q., Gao, C.X. and Qiu, J.L. (2017) Progress and prospects in plant genome editing. Nature Plant, 3, 1–6.

Yoshida, S., Forno, D.A., Cook, J.H. and Gomez, K.A. (1976) Routine procedures for growing rice plants in culture solution. In Laboratory Manual for Physiological Studies of Rice (Yoshida, S., Forno, D.A., Cook, J.H. and Gomez, K.A., eds), pp. 61–66. Los Banos, Philippines: International Rice Research Institute.

Zhang, Q. (2007) Strategies for developing green super rice. Proc. Natl. Acad. Sci. USA, 104, 16402–16409.

Zhang, L.Z., Tan, Q.M., Lee, R., Trethewy, A., Lee, Y.H. and Tegeder, M. (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in Arabidopsis. Plant Cell, 22, 3603–3620.

Zhao, H., Ma, H., Yu, L., Wang, X. and Zhao, J. (2012) Genome-wide survey and expression analysis of amino acid transporter gene family in rice (Oryza sativa L.). PLoS ONE, 7, e49210.

Zurcher, E. and Muller, B. (2016) Cytokinin synthesis, signaling, and function advances and new insights. Int. Rev. Cell Mol. Biol. 324, 1–38.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Effect of different NH₄NO₃ concentrations on growth of the rice seedlings with altered expression of OsAAP3.

Figure S2 The expression of OsAAP3 is regulated by both amino acids Lys and Arg.

Figure S3 Effect of different amino acids (Asp, Ser, Gly and Tyr) concentrations on outgrowth bud elongation of the rice seedlings with ZH11 and altered expression of OsAAP3.

Figure S4 Effect of different amino acids (Thr, Ala, Val, Leu and Gin) concentrations on outgrowth bud elongation of the rice seedlings with ZH11 and altered expression of OsAAP3.

Figure S5 Effect of different amino acids (Ile, Phe and His) concentrations on outgrowth bud elongation of the rice seedlings with ZH11 and altered expression of OsAAP3.

Figure S6 Effect of different amino acids Lys and Arg on growth of the rice seedlings with altered expression of OsAAP3.

Figure S7 The expression of OsCKXs in basal part of the rice seedlings with altered expression of OsAAP3 grown for 3 weeks in basic nutrient solution with 1.0 mM NH₄NO₃ as the N source.

Table S1 List of the primers in this study.