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Concurrent activation of *OsAMT1;2* and *OsGOGAT1* in rice leads to enhanced nitrogen use efficiency under nitrogen limitation

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SUMMARY

Nitrogen (N) is a major factor for plant development and productivity. However, the application of nitrogenous fertilizers generates environmental and economic problems. To cope with the increasing global food demand, the development of rice varieties with high nitrogen use efficiency (NUE) is indispensable for reducing environmental issues and achieving sustainable agriculture. Here, we report that the concomitant activation of the rice (*Oryza sativa*) *Ammonium transporter 1;2* (*OsAMT1;2*) and *Glutamate synthetase 1 (OsGOGAT1)* genes leads to increased tolerance to nitrogen limitation and to better ammonium uptake and N remobilization at the whole plant level. We show that the double activation of *OsAMT1;2* and *OsGOGAT1* increases plant performance in agriculture, providing better N grain filling without yield penalty under paddy field conditions, as well as better grain yield and N content when plants are grown under N limitations in field conditions. Combining *OsAMT1;2* and *OsGOGAT1* activation provides a good breeding strategy for improving plant growth, nitrogen use efficiency and grain productivity, especially under nitrogen limitation, through the enhancement of both nitrogen uptake and assimilation.

Keywords: nitrogen use efficiency, *Oryza sativa*, grain quality and yield, *Ammonium transporter 1;2*, *Glutamate synthetase 1*, activation tagging mutant.

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INTRODUCTION

The macronutrient nitrogen (N) is essential for plant growth and development because its shortage is critical for the synthesis of numerous important biological compounds such as amino acid, proteins, nucleic acids, chlorophyll and some plant hormones (Yang *et al.*, 2015). Therefore, plant productivity depends heavily on N fertilization and N is a major limiting factor in most agricultural practices (Kaur *et al.*, 2017). However, an excess input of fertilizer may result in serious concerns as a result of nitrate leakage in the field, leading to environmental pollution and an increase in economic cost (Li *et al.*, 2017). Therefore, reducing fertilizer usage at the same times as maintaining crop yield is one of the main goals of plant breeding (Masclaux-Daubresse and Chardon, 2011). Accordingly, it is very critical to enhance nitrogen use efficiency (NUE) to achieve sustainable crop production with the aim of meeting population growth and protecting the environment. NUE is a complex trait affected by both genetic and environmental factors. For crops, NUE is based on the plant seed yield and seed N concentration obtained per unit of available N in the soil (Perchlik and Tegeder, 2017; Cañas *et al.*, 2020). NUE depends on two key components. One is N uptake efficiency, which involves absorption/uptake of supplied N. The other one is N utilization efficiency, which involves assimilation and remobilization of plant N to grain (Huang *et al.*, 2017; Weih *et al.*, 2018). Therefore, high NUE crops are plants that can efficiently uptake, utilize and remobilize all of the N resource available to them (Li

et al., 2017; Selvaraj *et al.*, 2017). Consequently, understanding the various components for N uptake, assimilation and remobilization during a plant's life history is important for increasing NUE.

Nitrate (NO_3^-) and ammonium (NH_4^+) are the two main inorganic N forms in the soils. NH_4^+ is the major form available for rice grown in the paddy field and is the main inorganic N form found in the rice plant body (Tabuchi *et al.*, 2007). Ammonium ions are actively taken up by the roots via ammonium transporters (AMTs). There are at least 12 putative *OsAMT*-like genes in rice (Li *et al.*, 2012). Ammonium is assimilated into amino acid via the glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle (Xu *et al.*, 2012). GS catalyzes the formation of glutamine (Gln) condensing glutamate (Glu) and ammonia, and plays an essential role in the N metabolism (Xu *et al.*, 2012). Rice genome harbors three homologous genes for cytosolic GS (*OsGS1;1*, *OsGS1;2* and *OsGS1;3*) and one chloroplastic gene (*OsGS2*). *OsGS1;1* was expressed in all organs, and a lack of *OsGS1;1* resulted in severe retardation in growth rate and grain filling (Tabuchi *et al.*, 2005). GOGAT catalyzes the transfer of the amide group of Gln, formed by GS, to 2-oxoglutarate (2-OG) to release two molecules of Glu. There are two types of GOGAT, the ferredoxin-dependent (Fd-GOGAT) and NADH-dependent (NADH-GOGAT), which use ferredoxin and NADH electron donors, respectively (Lancien *et al.*, 2002). In rice, there are two NADH-type GOGATs and one Fd-GOGAT (Tabuchi *et al.*, 2007). The *OsNADH-GOGAT1* is mainly expressed in the root epidermis subsequent to the supply of NH_4^+, and is therefore responsible for the primary assimilation of ammonium at the root level (Yamaya and Kusano, 2014). The *OsNADH-GOGAT1* is also important in the development of new tillers in plants grown in paddy field (Tamura *et al.*, 2010).

Approaches aiming to improve NUE and crop productivity have mainly adopted the genetic manipulation strategy of modifying genes involved in N uptake, N allocation, and N metabolism and their regulation (Tegeder and Masclaux-Daubresse, 2018). For example, the overexpression of the nitrate transporter gene *OsNRT1.1A/OsNPF6.3* improves N utilization, increases grain yield and shortens maturation times (Wang *et al.*, 2018). Transgenic lines overexpressing *OsAMT1;1* have a significantly greater uptake capacity that improves overall plant growth and grain yield, especially under suboptimal NH_4^+ levels (Ranathunge *et al.*, 2014). As a result of the central role of GS in N metabolism, GS genes are major targets for genetic manipulation, and overexpressing different GS isoforms has been tested in many plant species with the aim of increasing N utilization (James *et al.*, 2018). By contrast to GS, GOGAT has been poorly manipulated in plant engineering (Xu *et al.*, 2012), although a metaQTL analyses performed in wheat revealed that GOGAT plays strategic role in NUE (Quraishi *et al.*, 2011).

In the present study, we isolated the double activation tagging lines generated by gene pyramiding that enhance simultaneously the expression of the *OsAMT1;2* and *OsGOGAT1* genes. We investigated the impact of the concomitant activation of both genes on grain yield and NUE traits under N-limited growth conditions and show that such plant engineering significantly improves the performance of rice and is a valuable breeding strategy.

RESULTS

Isolation of activation tagging mutants and generation of double activation mutants

To determine whether the enhanced expression of *OsAMT1;2* (LOC_Os02g40710) and *OsGOGAT1* (LOC_Os01g48960) affect plant growth and N utilization, we isolated the activation tagging lines from the rice flanking-sequence tag database (An *et al.*, 2003; Jeong *et al.*, 2006). Two independent T-DNA activation tagging mutants of *OsAMT1;2* were isolated; in these mutants, the 35S 4× enhancer elements were inserted in approximately 6.0 kb (*OsAMT1;2-D1*) and 5.5 kb (*OsAMT1;2-D2*) upstream from the *OsAMT1;2* coding region (Figure S1a). These lines were confirmed as activation tagging lines based on the increased expression of *OsAMT1;2* transcripts, as shown by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis in N-sufficient and -limited roots, compared to wild-type (WT) (Figure S1b). We also identified two activation tagging lines of *OsGOGAT1*; in these lines, the enhancer elements were inserted in approximately 0.9 kb (*OsGOGAT1-D1*) and 2.3 kb (*OsGOGAT1-D2*) upstream of the ATG start codon of the *OsGOGAT1* (Figure S1c). A subsequent qRT-PCR revealed that transcript levels of *OsGOGAT1-D1* and *OsGOGAT1-D2* were increased compared to WT at the seedling stage (Figure S1d).

To investigate the effects on the plant growth caused by the simultaneous enhanced expression of *OsAMT1;2* and *OsGOGAT1*, we then generated the double activation mutants by crossing the *OsAMT1;2-D2* and *OsGOGAT1-D1* lines because these two lines showed higher expression levels compared to the others.

Activation tagging mutants showed the enhanced tolerance to N limitation at the seedling stage

To investigate the effect of the enhanced expression of *OsAMT1;2* or *OsGOGAT1* under N limitation at the seedling stage, plants were grown on the modified Murashige and Skoog (MS) medium in which there were different concentrations of NH_4^+ as the N source. When grown in N-sufficient medium containing 10 mM NH_4^+, the growth of activation tagging mutants was comparable to that of WT, as shown by a similar plant height, biomass and chlorophyll concentration (Figures 1a,b and S2a,b). When grown in N-limited medium containing 0.1 mM NH_4^+, both WT and activation tagging lines showed shorter plant height,

as well as less biomass and chlorophyll, compared to growth in N-sufficient medium (Figures 1a,b and S2a,b). However, in N-limited medium, activation tagging mutants exhibited greater plant height (112.6–135.6% of WT) and biomass (110.8–123.9% of WT) than WT, showing the enhanced tolerance to N limitation of activation tagging mutants (Figures 1a,b and S2a). Accordingly, activation tagging mutants accumulated more chlorophyll than WT plants (117.9–122.6% of WT) (Figure S2b).

When grown under N-sufficient conditions, activation tagging lines accumulated similar levels of N in shoots (Figure 1c) but higher N levels in roots (7.3–11.6% higher than WT; Figure 1c). Under N limitation, mutants contained more N in both shoots and roots, which could explain their better tolerance to low N (Figure 1a,d). The shoot N concentrations of *OsAMT1;2-D1*, *OsAMT1;2-D2*, *OsGOGAT1-D1*, *OsGOGAT1-D1* and double activation mutants were 111.0, 113.8, 122.0, 131.2 and 115.6% of that of WT, respectively (Figure 2d); the root N concentrations of *OsAMT1;2-D1*, *OsAMT1;2-D2*, *OsGOGAT1-D1*, *OsGOGAT1-D1* and double activation mutants were 119.5, 126.8, 115.6, 117.1 and 120.7% of that of WT, respectively (Figure 2d). Under N-sufficient conditions, the shoot and root protein concentrations were similar in WT and activation tagging lines (Figure S2c), whereas mutants accumulated more protein in shoots (108.8–121.7% of WT) and roots (109.1–113.5% of WT) under N limitation (Figure S2d).

Because plant growth of activation tagging lines was better than WT under N limitation, this suggested better ammonium assimilation in these genotypes. We then monitored the expression and activity of GS. Expression of the two major cytosolic GS, *OsGS1;1* and *OsGS1;2* was higher in mutant seedlings than in the WT irrespective of external N status (Figure 1e,f), suggesting better N-assimilation in activation tagging lines. The expression levels of these two genes were even higher in the double mutant than in single mutants (Figure 1e,f). Accordingly, GS activities in shoots and roots were higher in the activation tagging mutants than in WT irrespective of N conditions (Figure 1g) and the GS activity of the double mutant was the highest recorded amongst the different genotypes. There were no differences in the expression of *OsGS2* and *OsGOGAT2* (Figure S2e,f), thus suggesting that these two genes played minor roles in the better performance of the activation tagging lines.

Nitrogen uptake is increased in activation tagging mutants

To test the effects of the enhanced expression of *OsAMT1;2* and *OsGOGAT1* on N uptake, we conducted ^{15}N

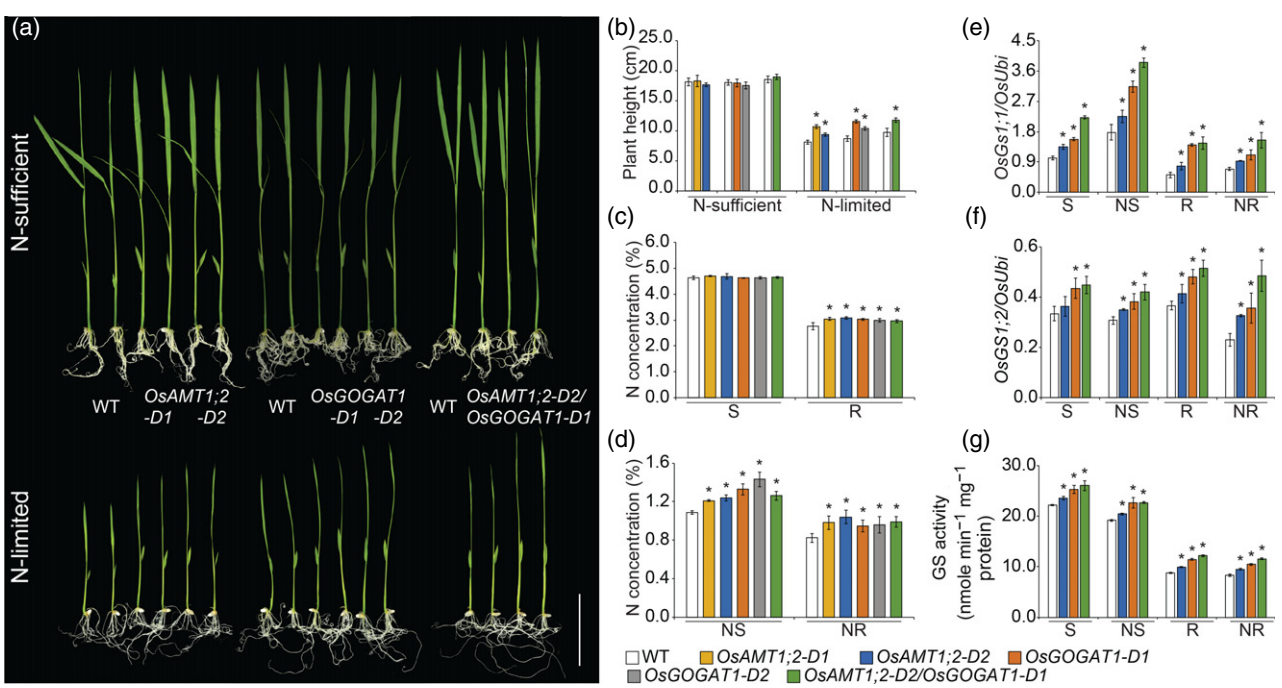

Figure 1. Activation tagging mutants show enhanced tolerance to nitrogen (N) limitation at the seedling stage.
(a) Phenotypes of the wild-type (WT) and activation tagging mutant plants grown for 8 days on N-sufficient or N-limited media. Scale bars = 2.5 cm.
(b) Plant height of seedlings measured after 8-day growth under N-sufficient or N-limited media. Nitrogen concentrations in the shoots and roots of the WT and transgenic seedlings grown under N sufficiency (c) or N limitation (d).
S, shoot grown in N sufficiency; R, roots grown in N sufficiency; NS, shoots grown in N deficiency; NR, roots grown in N deficiency. Expression levels of *OsGS1;1* (e) and *OsGS1;2* (f) at seedling stage under different N supply.
(g) Glutamine synthetase activities in shoots and roots from WT and transgenic seedlings (8 days old). Values are the mean ± SE (n = 4). Asterisks indicate significant differences between genotypes as determined using Student's t tests. *$P < 0.05$.

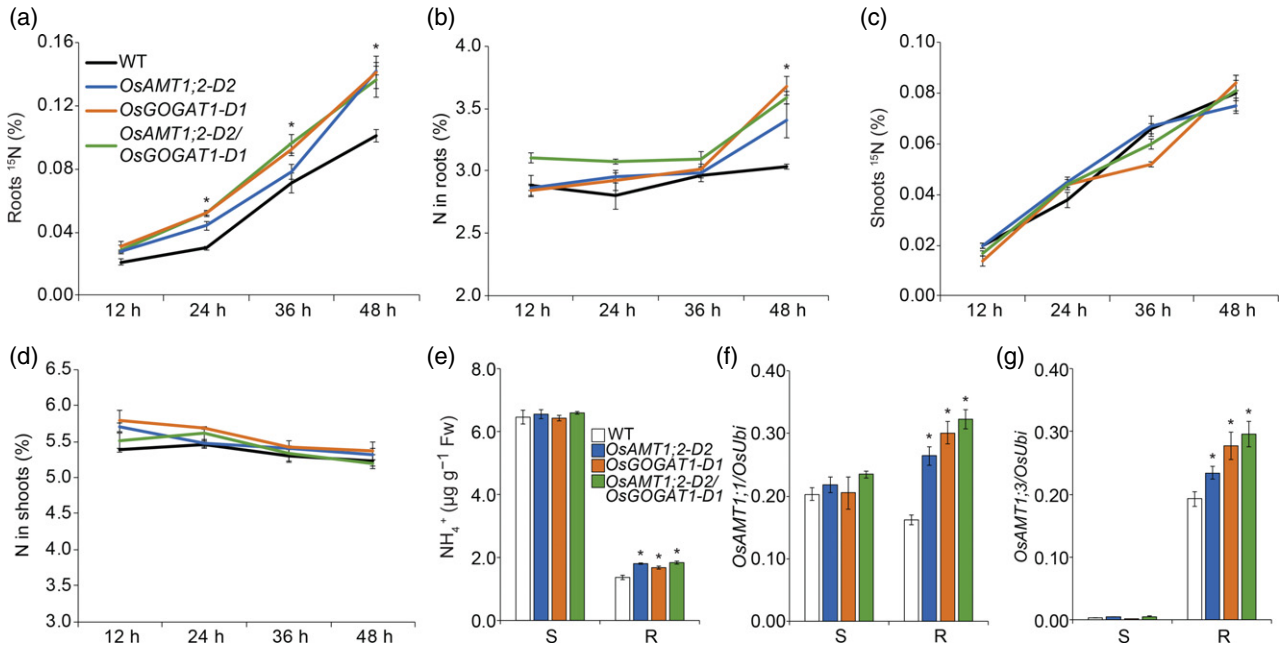

Figure 2. Nitrogen (N) uptake is enhanced in activation tagging mutants. Wild-type (WT) and activation tagging mutant seedlings were grown in the Yoshida solution for 7 days, and then shoots and roots were harvested 12, 24, 36 and 48 h after transfer from unlabeled NH_4Cl to ^{15}N-labeled NH_4Cl. ^{15}N concentrations (a, c) and total nitrogen (b,d) of roots (a,b) and shoots (c,d) from WT and activation aging mutants.
(e) Measurement of NH_4^+ in shoots and roots grown in hydroponic culture for 9 days. Quantitative reverse transcriptase-polymerase chain reaction expression analysis of *OsAMT1;1* (f) and *OsAMT1;3* (g) in shoots and roots of WT and activation tagging mutants under normal hydroponic cultivation for 9 days.
S, shoots; R, roots. Values are the mean ± SE of four randomly selected plants. Asterisks above the bars indicate significant differences between mutants and WT. $*P < 0.05$.

labeling using 7-day-old seedlings grown in hydroponic culture (Figure 2). Shoots and roots were harvested 12, 24, 36 and 48 h after ^{15}N treatment. ^{15}N uptake in root was higher in all the activation mutants than in WT (Figure 2a) and the faster uptake was measured in the *OsGOGAT1-D1* and the *OsAMT1;2-D2/OsGOGAT1-D1* lines. Accordingly, total N concentrations in the roots of mutants were higher than in those of WT (Figure 2b). Furthermore, ammonium concentrations in the mutant roots were higher than in WT (Figure 2e). By contrast to roots, there was no difference in the concentrations of ^{15}N, total N and ammonium in the shoots of WT and mutant seedlings (Figure 2c,d). The expression levels of *OsAMT1;1* and *OsAMT;3* were increased in the roots of mutant seedlings, which was in good accordance with the higher N uptake measured in the mutant roots using $^{15}NH_4^+$ (Figure 2f,g).

To confirm that the transporters involved in ammonium uptake were more active in the activation tagging lines, we subjected plants grown on solid medium to methylammonium (MeA). MeA is an ammonium analog that is toxic to yeast and plants and AMT proteins facilitate its uptake (Loqué *et al.*, 2006; Yuan *et al.*, 2007; Pantoja, 2012). MeA strongly inhibited the growth of WT and mutant seedlings in a dose-dependent manner (Figure S3a). However, mutants clearly exhibited increased sensitivity to MeA treatment compared to WT as shown by the higher inhibition of plant growth, which was monitored measuring plant height and biomass (Figure S3). This confirmed that the transporters involved in ammonium uptake were more active in mutant plants than in WT.

Nitrogen remobilization efficiency is higher in the *OsGOGAT1-D1* and double activation tagging lines

Previous reports indicated that the N is always distributed mostly to new growing organs regardless of growth stages of rice (Mae, 1986) and *OsGOGAT1* is important for the reutilization of N in developing organs (Tabuchi *et al.*, 2007). Therefore, we compared the effect N translocation from old leaves to newly emerging leaves in WT and mutant plants at the vegetative stage via a ^{15}N tracing assay. Plants were labelled with $^{15}NH_4Cl$-containing Yoshida solution until the seventh leaf stage from germination (time T_0). Labeled roots and leaves were harvested at T_0 from five plants per genotype. The remaining five plants were transferred to ^{15}N free solution and were grown until the tenth leaf stage over 3 weeks (time T_1 is the end of chase period). At T_1, roots, old leaves previously labeled at T_0 (from the first to seventh leaves) and leaves newly emerged after T_0 (eight to tenth leaves) were harvested separately for ^{15}N analysis. At T_0, there were no significant differences in the ^{15}N distribution patterns between WT and mutant plants (Figure 3a). At T_1, ^{15}N partition patterns were similar in WT and *OsAMT1;2-D2* plants

(Figure 3b). Interestingly, at T_1, the partition of ^{15}N in the new leaves was significantly higher in the *OsGOGAT1-D1* and double *OsAMT1;2-D2*/*OsGOGAT1-D1* than in WT and *OsAMT1;2-D2* (Figure 3b). This showed that the translocation of ^{15}N from the old leaves to newly formed leaves was more efficient in the mutants carrying the *OsGOGAT1-D1* construct.

Activation tagging mutants grown in paddy fields display improved N grain filling and better N remobilization from flag leaf

When grown in a conventional paddy field, we observed that the single and double activation tagging mutants exhibited earlier leaf senescence compared to WT (Figure S4a,b). At the heading stage, mutants and WT had similar levels of chlorophyll, whereas, after grain filling, chlorophyll contents were lower in the leaves of mutants than in that of WT (Figure S4a,b). In accordance with the leaf senescence phenotype, the expression of senescence upregulated genes, *OsSGR* (Park *et al.*, 2007) and *OsNAP* (Liang *et al.*, 2014) was higher in the mutants than those in WT (Figure S4c,d). Despite the difference in senescence onset, we did not note any difference in flowering time between activation mutants and WT plants. Because leaf senescence was more precocious in mutants, we focused our further analyses on the flag leaves and measured their N concentration during grain filling and at the senescence stage. At 3 weeks after heading, activation tagging mutants contained more N in the flag leaves than WT (Figure 4a). Because they contained a similar amount of soluble proteins and free amino acids (Figure 4b,c), we concluded that the difference in N content was a result of inorganic N or insoluble proteins. At the later grain filling stage (7 weeks after heading), N concentration in flag leaves was lower than at heading stage (Figure 4a). This was a result of the remobilization of N from the flag leaf to the grain. Interestingly the decrease in N% between the early and later grain filling stages was stronger in the single and double mutants than in WT, and both protein and amino acid levels were lower in the mutants than in the WT (Figure 4a–c). The expression analysis of *OsGS1;1* and *OsGS1;2* in flag leaves indicated that their levels were higher in mutants than in WT at the grain filling stage; accordingly, GS activities was higher in mutants (Figure 4d–f). This confirmed that N remobilization was better in mutants than in WT. Among mutants, the double mutant showed the highest *OsGS1;1* and *OsGS1;2* expression levels and the highest GS activity, which is consistent with the previous results conducted at the seedling stage. The expression levels of *OsGS2* and *OsGOGAT2* in flag leaves of WT and mutants (Figure S5) were similar to each other, as at the seedling stage.

When grown in the field, plant height and aboveground dry weight of the single activation tagging mutants (*OsAMT1;2-D1*, *OsAMT1;2-D2*, *OsGOGAT1-D1, OsGOGAT1-D2*) were significantly lower than that of WT (Figures 5a and S6a,b). Single activation tagging mutants showed a reduced spikelet number per panicle and lower fertility, resulting in lower productivity compared to WT (Figures 6b,c and S6c,d). The number of tillers was similar to that in WT plants (Figure S6e). As a result of the reduced grain length, thickness and width, the seed weight was also lower in mutants compared to WT (Figures 5d and S6f–h).

Interestingly and unexpectedly, growth and morphology of the double *OsAMT1;2-D2*/*OsGOGAT1-D1* activation tagging mutant grown in a paddy field were comparable to

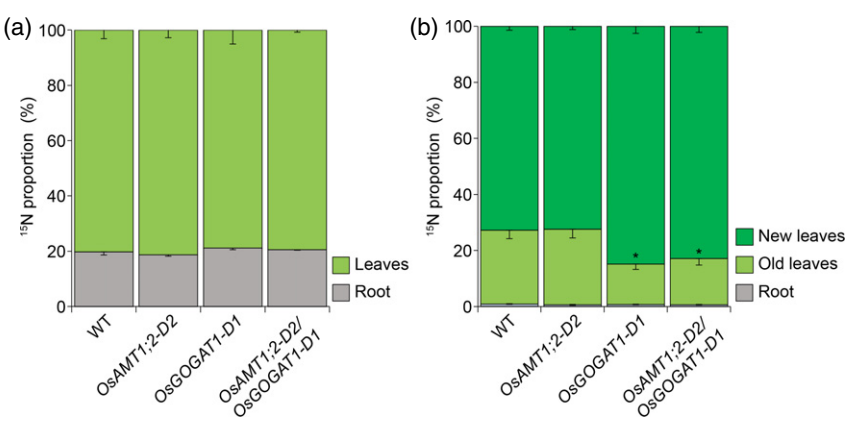

Figure 3. Nitrogen remobilization efficiency is increased in the *OsGOGAT1-D1* single and double activation tagging lines. Hydroponically grown wild-type (WT) and activation tagging mutants were labelled using ^{15}N-labeled NH_4Cl containing Yoshida solution until the seventh leaf stage (time T_0). Labeled roots and leaves were harvested from five plants. The remaining plants were transferred to Yoshida solution containing $^{14}NH_4Cl$ instead of $^{15}NH_4Cl$, and grown over 3 weeks until the tenth leaf stage (time T_1; the chase period). Then roots, previously labeled old leaves (from first to seventh leaves) and newly formed leaves (eight to tenth leaves) were harvested separately for ^{15}N analysis.
(a) Partitioning patterns of ^{15}N in roots and leaves of WT and mutant plants at the seventh leaf stage (at T_0).
(b) Partitioning of ^{15}N at T_1 in roots, old leaves, and newly emerged three leaves during the chase period.
Data are the mean ± SE of four plants. Significant differences between the mutant and WT as determined by Student's *t* test are indicated. $*P < 0.05$.

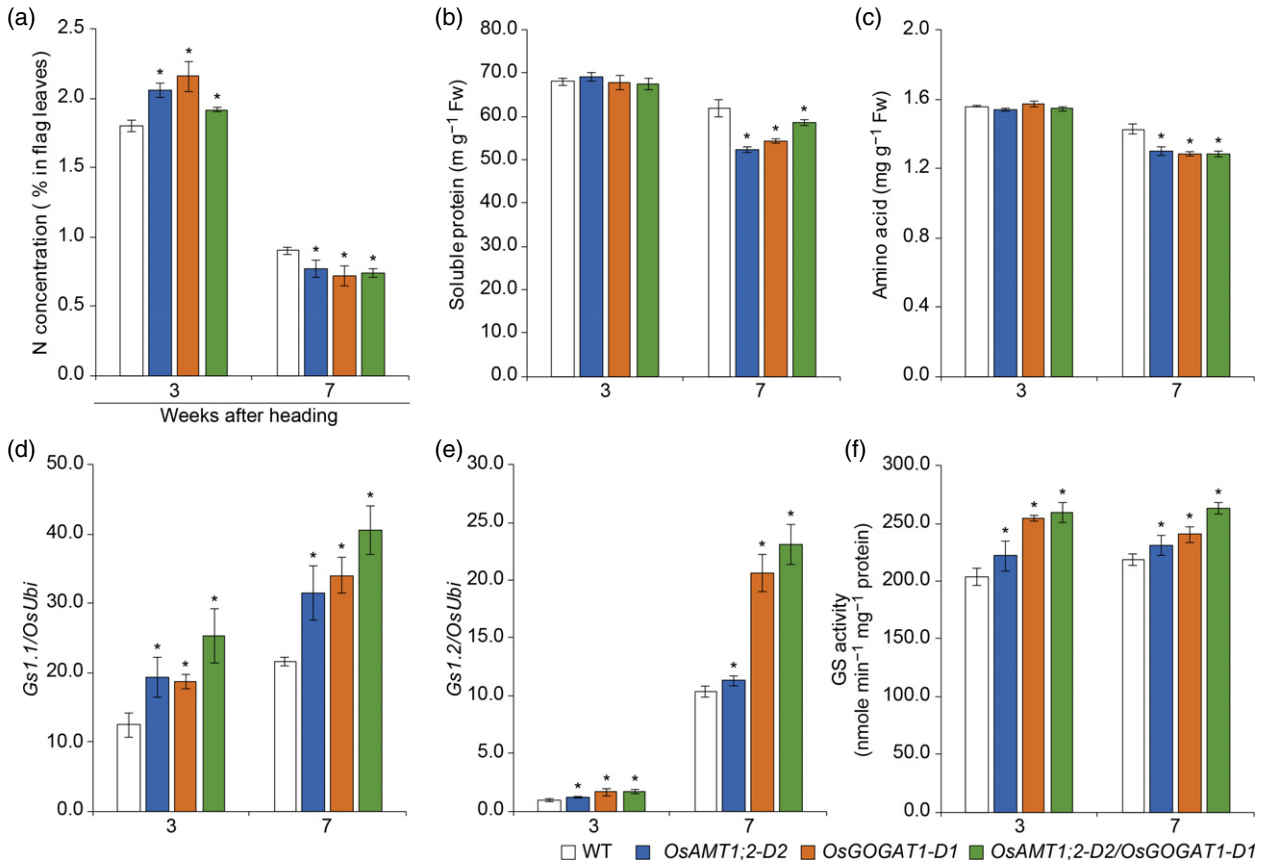

Figure 4. Activation tagging mutants display better nitrogen (N) remobilization from flag leaf.
(a) Measurement of nitrogen in flag leaves of wild-type (WT) and activation tagging mutants during grain filling periods (3 and 7 weeks after heading). Soluble protein (b) and amino acid (c) concentration in flag leaves. Expression analysis of *OsGS1;1* (d) and *OsGS1;2* (e) in flag leaves. (f) Glutamine synthetase activities of flag leaves.
Values are the mean ± SE (n = 4). Significant differences from WT were determined by Student's *t* tests; $*P < 0.05$.

those of WT (Figures 5a and S6a,b). Plant height, aboveground dry weight, number of spikelets per panicle, tiller number per plant and grain filling rates of double activation tagging mutants were similar to those of WT, leading to similar grain yield per plant (Figures 5a–c and S6a–e). The length, width and thickness of double mutant grains were also similar to those of WT grains (Figure S6f–h), resulting in no significant differences in grain weight between the WT and double mutant plants (Figure 5d).

The better performance of activation tagging lines was then revealed by the analyses of grain quality. Grains from activation tagging plants indeed accumulated more N than the WT grains. N concentration in the grains of *OsAMT1;2-D1*, *OsAMT1;2-D2*, *OsGOGAT1-D1*, *OsGOGAT1-D2* and double *OsAMT1;2-D2*/*OsGOGAT1-D1* mutants was increased to 115.5, 114.6, 141.7, 111.7 and 113.3% of that in WT grains, respectively (Figure 5e). Accordingly, protein and free amino acid concentrations in mutant grains were better than in WT (Figure 5f,g). The enhanced expression of *OsAMT1;2* or *OsGOGAT1* can therefore improve the nutritional quality of the grains of rice grown in conventional paddy fields. Such phenotypes of the activation tagging mutant and WT plants grown in paddy fields were confirmed in further experiments performed in subsequent years (Figure S7a–d).

Double activation improves grain yield under N-limiting conditions

Mutant and WT plants were grown in pots containing N-limited nursery soil (i.e. 25 mg/kg N until harvest). Although single activation tagging mutants displayed enhanced growth under N limitation at seedling stage, they did not show better growth and yield when N limitation was prolonged further until grain maturity (Figures 6a, b and S8). Agronomic traits of single mutants such as seed number per panicle, fertility, grain yield per plants and other traits were not improved under N limitation compared to WT (Figures 6a–d and S8). The 1000-seed weight from the single activation mutants was lower than that of WT (Figure 6e), although their grains accumulated more N and protein levels compared to WT (Figure 6f,g). By contrast, double activation tagging mutant displayed taller

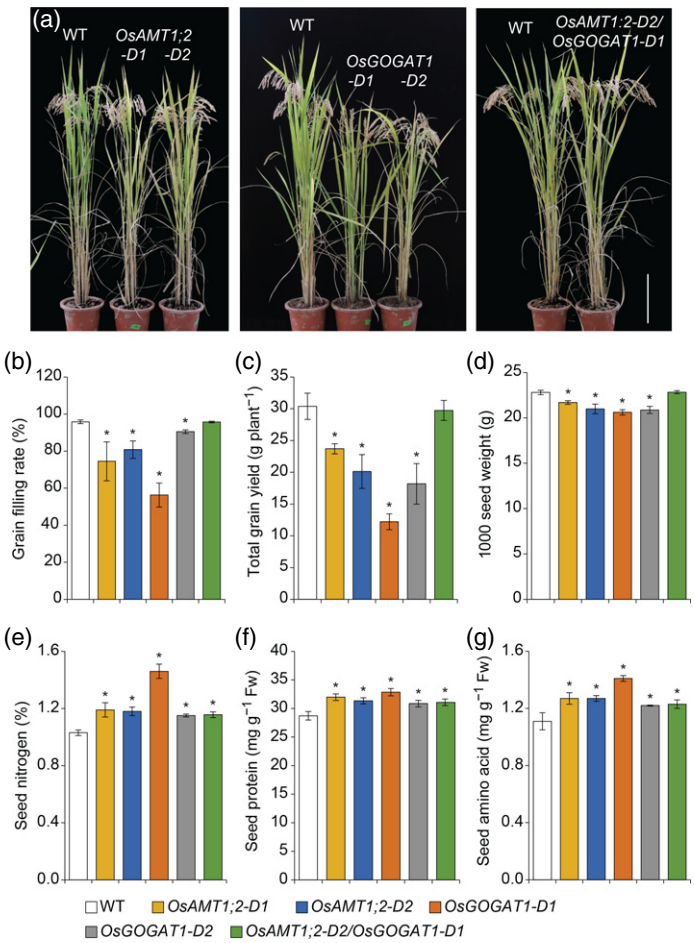

Figure 5. Activation tagging mutants display better nitrogen (N) seed filling when cultivated in paddy field.
(a) Representative morphology of wild-type (WT), *OsAMT1;2*- and *OsGOGAT1*-activation tagging mutants, and double mutant grown in a paddy field. Representative photographs were taken just before harvest (scale bar = 20 cm). Transgenic plants were transferred from the paddy field to pots for the photographs.
(b–d) Grain filling rate of WT and mutant plants (b), total grain yield per plants grown in the paddy fields (c), and 1000 seed weight (d) from WT and mutants.
(e–g) Nitrogen (e), protein (f), and amino acid (g) concentrations in grains. FW, fresh weight.
Data are the mean ± SE of five plants. Asterisks above the bars indicate significant differences between mutant lines and WT. $*P < 0.05$.

plants with improved growth under N limitation during the reproductive stage (Figures 6a–e and S8), as well as a significant increase in number of tiller and spikelets per panicle (Figure 6b,c), and thus improved grain yield (138.9% of WT; Figure 6d). The 1000-seed weight of the double mutant was comparable to that of WT (Figure 6e), although N concentration in its grains was higher (118.7% of WT; Figure 6f). Therefore, protein concentration in grains was enhanced to 115.0% of WT (Figure 6g). When the growth of the activation tagging plants was tested under outdoor N limitation growth conditions for a additional year, the double activation tagging mutant again displayed a higher grain yield and better N and protein concentrations in the grain (Figure S9). Overall, such data indicate that the double activation *OsAMT1;2/OsGOGAT1* leads to practical improvements in grain yield and grain protein concentrations also under N-limiting conditions.

DISCUSSION

Nitrogen macronutrient is a master factor for plant growth and development and N fertilizers represent an important cost in crop production. We have known for some time that improving plant NUE is essential for human and animal feeding and decreasing the negative impact of N fertilizer production on both the environment and the farmer's budget.

In the present study, we show that concurrent activation of *OsAMT1;2* and *OsGOGAT1* significantly increases NUE in rice and provides many positive effects on agronomical traits, including enhanced tolerance to N limitation and improved grain yield and grain protein content.

Because rice nitrogen preference is for ammonium, we chose to over-induce one member of the OsAMT1 ammonium transporter. The OsAMT1 subfamily includes

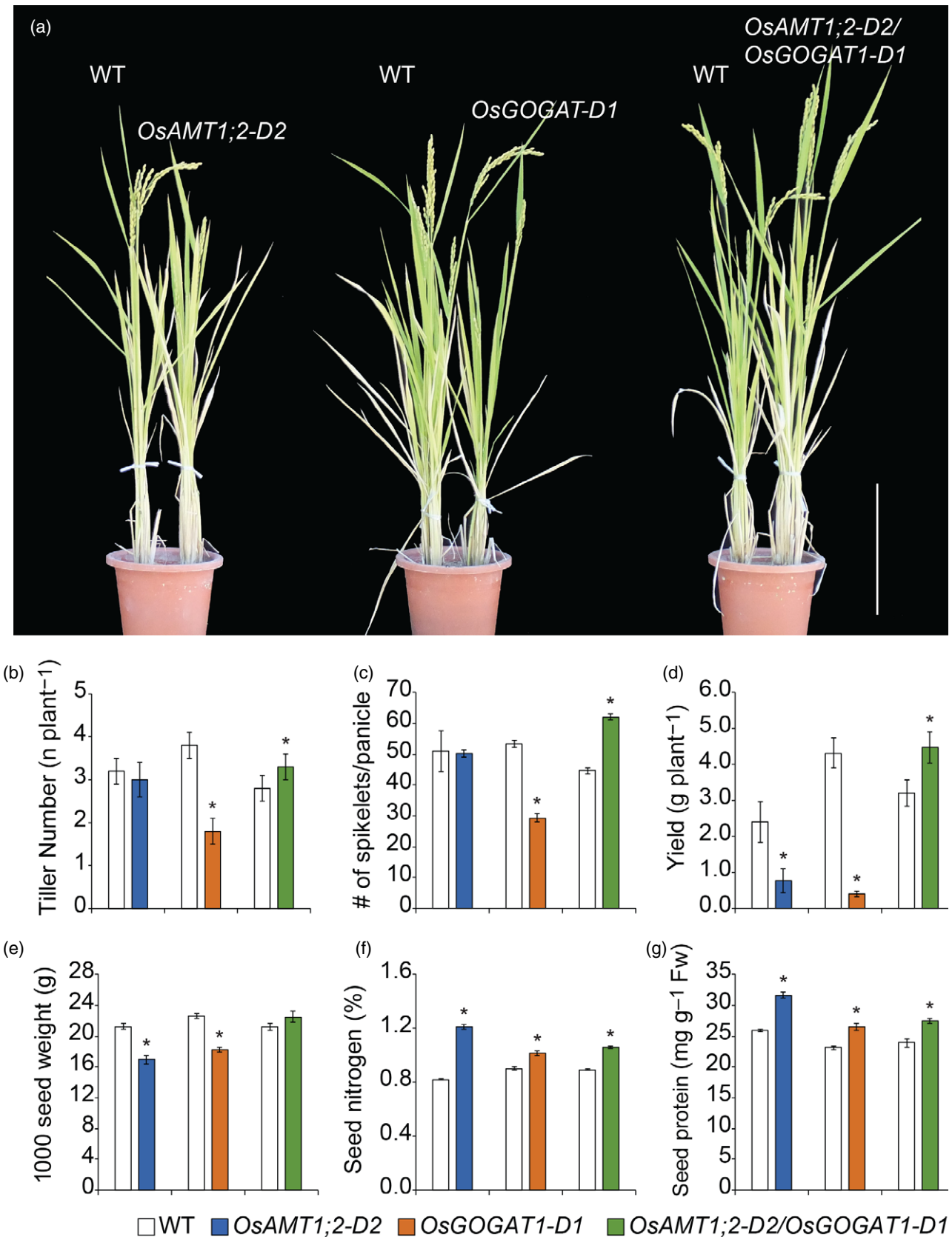

Figure 6. The concurrent activation of *OsAMT1;2* and *OsGOGAT1* in rice improves grain nitrogen (N) content without yield penalty under N-limiting conditions.
(a) Comparison of morphology of transgenic and wild-type (WT) plants grown under N limitation at the reproductive stage. Plants were grown in an outdoor condition until harvest in pots containing the conventional nursery soil with N deprivation comprising 1:16 level of the regular N-enriched nursery soil. Representative photographs were taken after 5 weeks after flowering. Scale bars = 20 cm.
(b) Tiller numbers of WT and activation tagging mutants.
(c) Number of spikelets per panicle of WT and transgenic plants.
(d) Total grain yield per plant.
(e) 1000 seed weight. Nitrogen (f) and protein (g) concentrations in mature seeds for plants as grown in N limitation. Error bars represent the SE of five replicates. Asterisks above the bars indicate significant differences between mutants and WT. $*P < 0.05$.

OsAMT1;1, OsAMT1;2 and OsAMT1;3. All of them have been characterized as high affinity ammonium transporters, meaning that they should facilitate ammonium uptake when ammonium is scarce. These three genes show distinct expression patterns (Sonoda *et al.*, 2003). *OsAMT1;1* is constitutively expressed in shoots and roots. *OsAMT1;2* is expressed in the central cylinder and cell surface of root tips and is ammonium-inducible. *OsAMT1;3* is root-specific and nitrogen-derepressible (Sonoda *et al.*, 2003). There were two contrasting results of overexpressing of *OsAMT1;1*. The constitutive expression of *OsAMT1;1* under the control of the maize *ubiquitin* promoter displayed a decreased biomass of transgenic lines during seedling and early vegetative stage as a result of the inability of ammonium assimilation to match the greater ammonium uptake (Hoque *et al.*, 2006). By contrast, overexpression of *OsAMT1;1* under the control of the maize *ubiquitin* promoter was shown to result in greater uptake capacity than the WT and also improved overall plant growth and grain yield, especially under suboptimal NH_4^+ levels (Ranathunge *et al.*, 2014). Kumar *et al.,* (2006) showed that the overexpression of *OsAMT1;1* under the control of the *CaMV-35S* promoter increased ammonium uptake capacity. The overexpression of *OsAMT1;3* was also tested and resulted in significant decreases in growth with poor nitrogen uptake ability, accompanied by a higher leaf C/N ratio (Bao *et al.*, 2015).

There is no report available so far on the effect of Os*AMT1;2* overexpression. As a result of its capacity to be inducible by ammonium, we chose to isolate activating tagging mutants of *OsAMT1;2* and found two independent lines (Figure S1a,b). The choice of activation tagging system is a result of the fact that, when using an enhancer(s) of the endogenous gene, the expression of the tagged gene can be increased often with little effect on the organ-specific expression pattern (Jeong *et al.*, 2006). Thus, our activation tagging lines maintained their root-specific expression of *OsAMT1;2* with the enhanced expression level. The *OsAMT1;2* activation tagging lines showed increased ammonium uptake capacity compared to WT as shown by ^{15}N tracing and MeA assays (Figures 2a and S3). In the *OsAMT1;2* activation lines, expression of *OsGS1;1* and *OsSG1;2* was higher than that in WT. Accordingy, GS activity in these lines was also increased compared to that in WT (Figure 1e–g). Because the NH_4^+ level was increased by the activation of *OsAMT1;2,* the increased GS activity in the tagging mutants would have led to higher rates of assimilation of NH_4^+ to glutamine, avoiding NH_4^+ toxicity (Jian *et al.*, 2018). Our result is consistent with a previous report on *OsAMT1;1* overexpressing transgenic plants; these plants also displayed an enhanced expression of *OsGS1;1* and *OsGS2:1*, leading to higher amounts of glutamine (Ranathunge *et al.*, 2014).

However, leaf to leaf nitrogen remobilization efficiency was not changed (Figure 3), which, even in activation tagging lines, was a result of the expression of *OsAMT1;2* being restricted to the root. Growth at the reproductive stage and grain yield of *OsAMT1;2-D1/2* in paddy field were, however, lower than WT (Figure 5a–d), which could occur in overexpressing ammonium transporters as shown by Bao *et al.,* (2015). Nevertheless, the grain of the *OsAMT1;2-D1/2* mutants accumulated more nitrogen, protein and amino acids compared to WT (Figure 5e–g). The lower yield and higher N content in the seeds of the *OsAMT1;2-D1/2* mutants could have been a consequence of their earlier flag leaf senescence. Although *OsAMT1;2* activation tagging mutants showed a higher ammonium uptake capacity and better tolerance to N limitation at seedling stage (Figures 1 and 2), their grain and biomass yields were not improved when grown in N-limiting growth condition (Figure 6).

It is well known that NH_4^+ is assimilated into glutamine and glutamate through the GS/GOGAT cycle and further incorporated into other amides and amino acids (Miflin and Habash, 2002; Bao *et al.*, 2014). It is also known that the GOGAT activity is the bottle neck of this cycle and that, by comparison, GS activity is very efficient (Masclaux-Daubresse *et al.*, 2006). The overexpression of GS genes had been investigated as a major strategy for enhancing NUE in several plant species (Xu *et al.*, 2012). However, the results obtained have generally been inconsistent (Thomsen *et al.*, 2014). Rice overexpressing *OsGS1;1* or *OsGS1;2* showed a higher total GS activity with an increased soluble protein concentration in leaves and a higher total nitrogen content in the whole plant, whereas grain yields and total amino acids were decreased (Cai *et al.*, 2009). Overexpression of *GS1;2* in rice resulted in increased NUE under the growth chambers, although there was no significant difference between WT and overexpressor plants in the greenhouse or under field conditions (Brauer *et al.*, 2011). The

manipulation of GOGAT has also been assayed in rice. Transgenic *indica* rice overexpressing the *NADH-OsGOGAT1* gene from *indica* under the control of a promoter from *japonica* rice showed increased grain yield (Yamaya *et al.*, 2002). Previous reports indicated that OsGOGAT1 is critical in the primary assimilation of NH_4^+ in roots (Tabuchi *et al.*, 2007; Yamaya and Kusano, 2014). Knockout of *OsGOGAT1* affected seedling root growth and the development of active tiller numbers (Tamura *et al.*, 2010), confirming the importance of *OsGOGAT1* in nitrogen assimilation and management in rice. The two *OsGOGAT1* activation tagging lines that we analyzed in the present study (Figure S1c,d) displayed enhanced tolerance to N limitation (Figure 1a,b). *OsGOGAT1*-activation tagging mutants also displayed increased ammonium uptake and GS activity, as did the *OsAMT1;2* activation lines (Figures 1g and 2a). The *OsGS1;1* overexpressing transgenic rice has previously been reported to display a higher *OsGOGAT1* expression level, which led to the suggestion of a feedback regulation loop in the GS/GOGAT cycle for efficient NH_4^+ assimilation (Bao *et al.*, 2014). Consistent with the suggested feedback regulation mechanism, the enhanced expression of *OsGOGAT1* in our activation tagging lines led to increased *OsGS1;1* expression. To balance the GS/GOGAT cycle for N assimilation, the expression of *OsAMTs* was then increased to fulfill ammonium substrate requirements for assimilation (Lea and Miflin, 2003).

Furthermore, our activation tagging mutants showed better nitrogen remobilization efficiency than WT at both the vegetative and reproductive stages (Figures 3 and 4a). However, no effect on productivity was observed on the *OsGOGAT1* activation tagging lines when grown in a paddy rice field (Figure 5a–c) or under N-limiting growth conditions (Figure 6a–d), although, similar to the *OsAMT1;2* activation tagging mutants, they contained more nitrogen and protein in mature grains (Figures 5e–g and 6f,g).

It is also known that ammonium can be toxic to plants and that it might be important when modifying ammonium uptake to also modulate ammonium assimilation steps to ensure the right management of potential toxic ammonium accumulation *in planta* (Esteban *et al.*, 2016). The manipulation of any single gene in the nitrogen metabolic pathway may lead to undesirable pleiotropic effects, originating from post-transcriptional, translational and/or feedback regulation to maintain homeostasis (Kurai *et al.*, 2011; Gao *et al.*, 2019). The accumulation of unbalanced metabolic intermediates may also result in some negative effect, such as poor plant growth and decreased yield (Yu *et al.*, 2016), as shown on the *OsGS1;1* or *OsGS1;2* overexpressors (Bao *et al.*, 2014). Then, we decided to combine the *OsAMT1;2* and *OsGOGAT1* activation tagging in the same rice plant to allow comparison with single activation tagging lines and WT, and to avoid possible unbalance between ammonium uptake and assimilation. We aimed to avoid the undesirable phenotypes of the single *OsAMT1;2* and *OsGOGAT1* activation tagging lines.

At the seedling stage, the double activation line *OsAMT1;2-D2*/*OsGOGAT1-D1* displayed better tolerance to N limitation (Figure 1a,b), exhibiting better growth and higher ammonium uptake capacity (Figure 2a) than the WT. This was the first indication of the positive effect of pyramiding. Glutamine synthetases are known to be induced by ammonium (Ishiyama *et al.*, 2004; Masclaux-Daubresse *et al.*, 2005). The increased levels of ammonium uptake in the *OsAMT1;2-D2*/*OsGOGAT1-D1* mutant are certainly the cause of the enhanced the expression of *OsGS1;1* and *OsGS1;2*, which led to higher GS activity in the mutants. The enhancement of GS activity was in good agreement with the better NUE observed in mutants and with the better N remobilization capacity measured using ^{15}N tracing in *OsAMT1;2-D2*/*OsGOGAT1-D1* mutant (Figures 1e–g, 3 and 4d–f). Although the double mutant was morphologically similar to the WT and showed a similar yield when plants were grown in the conventional N-sufficient paddy field, the higher nitrogen and protein contents in their mature grains are the result of their better N remobilization efficiency. The higher N and protein contents in the *OsAMT1;2-D2*/*OsGOGAT1-D1* mutant constitute a valuable grain quality improvement (Figure 5e–g). Furthermore, the better grain yield measured in the double mutant compared to WT and single mutants when grown under N-limiting conditions is a substantial improvement of the plant NUE (Figure 6). When we generated the double activation mutants, we stacked *OsAMT1;2-D2* and *OsGOGAT1-D1* lines because these two lines displayed higher expression levels than others. Generation of different combinations of double mutant lines would be a key future plan.

Based on these results, we propose a model in which double activation of *OsAMT1;2* and *OsGOGAT1* could promote nitrogen uptake, assimilation and remobilization to the seeds (Figure 7). As a result of the increased NH_4^+ uptake, GS activity is increased in double mutants. The concomitant enhancement of GOGAT1 then contributes to better ammonium assimilation into amino acids without modifying the balance between glutamate and glutamine. The better performance of the double activation tagging line, by comparison with WT and single mutants, is certainly also linked to a better source with respect to sink N remobilization because glutamate and glutamine are important components of the phloem saps. The fact that the *OsAMT1;2-D2*/*OsGOGAT1-D1*double mutant outperformed both WT and the single mutants demonstrates that this combination did not unbalance N uptake and assimilation in the wrong way, and also that it was appropriate for significantly improving NUE and especially grain protein contents in paddy fields.

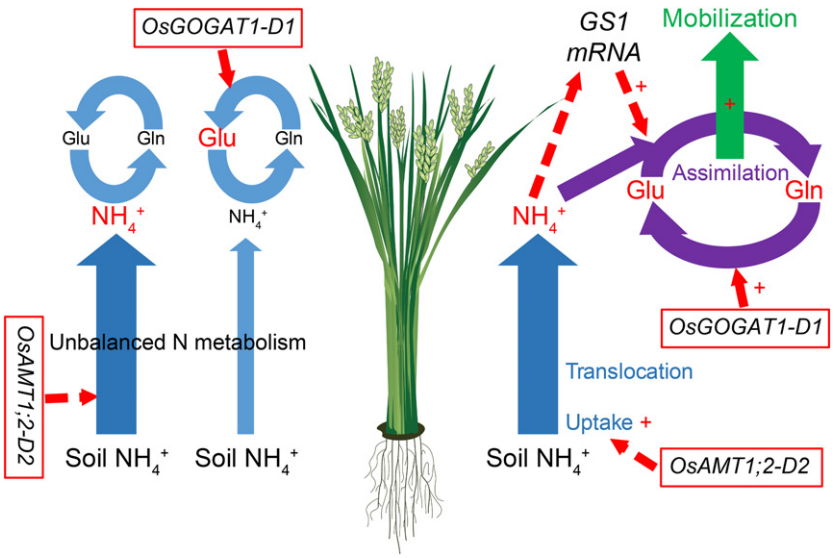

Figure 7. Schematic model of the concurrent activation of *OsAMT1;2* and *OsGOGAT1* in rice plants for the improvement of nitrogen use efficiency. Increased nitrogen (N) uptake and translocation in double mutants enhanced the expression of *OsGS1;1* and *OsGS1;2*, and thereby modified the nitrogen assimilation and remobilization, leading to the improved nitrogen use efficiency, grain protein contents and yields, especially under the constrained N fertilization. Positive effects of *OsAMT1;2-D2*, *OsGOGAT1-D1* and *OsAMT1;2-D2*/*OsGOGAT1-D1* are shown in red. Glu, glutamate; Gln, glutamine.

The present study demonstrates that simultaneously enhancing the expression of *OsAMT1;2* and *OsGOGAT1* can increase nitrogen uptake and utilization efficiencies in rice, as well as modulate other genes related to N uptake and metabolism, such as cytosolic GS. Consequently, the double activation of *OsAMT1;2* and *OsGOGAT1* can confer a better growth and improve the grain yield and quality of rice, especially under N limitation, and thus constitutes a promising strategy for improving the NUE of rice plants and multiple crop species.

EXPERIMENTAL PROCEDURES

Plant growth

For the growth of WT and the transgenic activation tagging lines, seeds were germinated on a modified half-strength MS agar medium at 28 °C under continuous light. To test the effect of N limitation at seedling stage, we germinated seeds and then grew seedlings for 8 days on a modified 1/2 MS media containing 0.1 mM NH_4Cl as the only N source, and 5 mM KCl instead of 5 mM KNO_3. For nitrogen sufficiency, 10 mM NH_4Cl added to modified MS medium as N source, and KCl was substituted for KNO_3. For the sensitivity test to MeA, WT and transgenic seedlings were grown for 8 days on a modified 1/2 MS solid media containing 0, 50 or 75 mM MeA.

At 30 days after sowing, WT and transgenic seedlings were transplanted at a spacing of 15 × 30 cm and grown to maturity in paddy fields located at Daegu Gyeongbuk Institute of Science and Technology (36° N) in 2017 and 2018 under natural conditions. Approximately 20 plants from each line were planted and each set of experiments was repeated twice. Fertilizer was applied as described previously (Oh *et al.*, 2009). For evaluation of the N limitation at the reproductive stage, four-leaf stage seedling of WT and activation tagging mutants were grown in the same pot (diameter 17 cm, height 18 cm) containing the N-limited nursery soil with 1:16 the level N of regular, N-enriched nursery soil (400 mg/kg) under outdoor growth condition until harvest. Yield parameters were scored in five plants of each genotype including plant height, aboveground mass, spikelet number per panicle, tiller number per plant, filling rate, grain yield per plant, 1000-grain weight, seed length, seed thickness and seed width.

RNA preparation and mRNA quantification

Total RNA was isolated from frozen samples with WelPrep total RNA isolation reagent (WELGENE, Gyeongsan, Republic of Korea), in accordance with the manufacturer's instructions and treated with RNase-free DNase I (Takara Bio, Shiga, Japan) to prevent genomic DNA contamination. For first-strand cDNA synthesis, we used 2 μg of total RNA in a 25-μL reaction mixture with using the ImProm II Reverse Transcriptase system kit (Promega, Madison, WI, USA). qPCR analysis was performed to determine gene expression levels (CFX96 Touch Real-Time PCR Detection System; Bio-Rad, Hercules, CA, USA) using a SYBR premix ExTaq kit (Takara Bio). The levels of *OsUbi* mRNA served to normalize the expression ratio for each gene. Changes in expression were calculated via the $\Delta\Delta C_t$ method. Gene-specific primers used for qRT-PCR are listed in Table S1.

Chlorophyll quantification and determination of total nitrogen, soluble protein and free amino acids

The chlorophyll contents of flag leaves from the main culm were estimated using a CCM-300 chlorophyll meter (Opti-Sciences, Hudson, NH, USA). To measure total nitrogen, samples from WT and mutants were dried at 80°C for 3 days and then were ground to a fine powder with a mortar and pestle. Determination of total N contents was determined by Elemental Analyzer (Vario MICRI cube; Elementar, Langenselbold, Germany) in accordance with the manufacturer's instructions. Acetanilide was used as a standard. Amino acids were extracted using 2% (w/v) sulfosalicylic acid and quantified as described previously (Rosen, 1957).

Measurement of NH_4^+ and GS activity

For free NH_4^+ analysis, plant materials were homogenized by grinding in cold 2% 5-sulfo salicylic acid and then the homogenates were centrifuged at 12 000 ***g*** for 20 min at 4°C. Free NH_4^+ in the supernatant was determined by using Berthelot color reaction method (Rhine *et al.*, 1998). Absorbance at 635 nm was determined and NH_4^+ contents were calculated from the standard curve of $(NH_4)_2SO_4$. For the total GS enzyme activity assay, leaves from WT and mutants were homogenized in a GS extraction buffer containing 100 mM Tris-HCl (pH 7.6), 1 mM $MgC1_2$, 1 mM ethylenediaminetetraacetic acid (EDTA) and 14 mM 2-mercaptoethanol. The homogenate was centrifuged at 16 200 *g* for 15 min at 4 °C. Assays were performed using on crude extracts after centrifugation, by incubating with samples with reaction buffer (buffered at pH 7.6) containing glutamate (600 mM), $MgCl_2$ (150 mM), EDTA (30 mM), NH_2OH (6 mM), ATP (60 mM) and Tris-HCl (50 mM). After 30 min of incubation, the reaction was stopped adding a solution containing $FeCl_3$, TCA and HCl to obtain as final concentrations in the reaction mix 185 mM, 100 mM and 2.75% of these compounds, respectively. Blank was obtained by omitting ATP in the reaction buffer. After centrifugation, the optical density of mixes was measured spectrometrically at 540 nm. Values of GS activity were extrapolated from a standard calibration curve made from different known concentrations of γ-glutamylhydroxamate (Sigma-Aldrich, St Louis, MO, USA). One unit of GS activity represents 1 μmol of γ-glutamylhydroxamate produced in 30 min.

^{15}N labeling for the uptake analysis and measurement of remobilization efficiency

For ^{15}N uptake analysis at seedling stage, seedlings of WT and activation tagging mutants were cultured in the Yoshida nutrient solution for 7 days (Yoshida *et al.*, 1976), except that 1.44 mM NH_4NO_3 was replaced by 2.88 mM NH_4Cl. Next, seedlings were treated with ^{15}N-labeled NH_4Cl (10% atom $^{15}NH_4Cl$; Sigma-Aldrich). Roots and shoots were harvested at the time of 12, 24, 36 and 48 h after treatment and dried at 70°C.

To monitor ^{15}N remobilization efficiency, seeds were germinated and cultured in the Yoshida solution containing ^{15}N-labeled 2.88 mM NH_4Cl (10% atom $^{15}NH_4Cl$; Sigma-Aldrich) until the emergence of the seventh leaves (time T_0). At T_0, labeled root and leaf samples were harvested from five plants. The remaining plants were transferred to unlabeled nutrient solution and grown until the tenth leaf stage (time T_1; chase period). At T_1, roots, old leaves previously labelled (from first to seventh leaves) and newly emerged leaves (eighth to tenth leaves) were harvested from five plants. After drying and weighing, samples were ground and ^{15}N abundance was estimated as described previously (Masclaux-Daubresse and Chardon, 2011).

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

SL, CM-D and HGN conceived the study and designed the research plan. SL, JP and T-HK carried out field work and phenotyping. AM, JP, CF, YY and SK provided technical assistance for the analyses the mutants. SL, CM-D, POL and HGN wrote the paper. All the authors read and approved the final version of the manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Molecular characterization of activation tagging mutants.

Figure S2. Growth of WT and activation tagging mutants grown under N sufficiency or N limitation.

Figure S3. Sensitivity to methylammonium (MeA) is enhanced in the activation tagging mutants.

Figure S4. Leaf senescence phenotype of WT and mutants.

Figure S5. Expression analysis of *OsGS2* and *OsGOGAT2*.

Figure S6. Characterization of activation tagging mutants grown in paddy fields.

Figure S7. Phenotypes of activation tagging mutants grown in paddy field in 2018.

Figure S8. Characterization of WT and activation tagging mutants under nitrogen limitation at the reproductive stage.

Figure S9. Growth test of WT and activation tagging mutants under nitrogen limitation at the reproductive stage in 2018.

Table S1. List of primers used in the present study.

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