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 time 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; Caracas 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).
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 (\(\text{NO}_3^-\)) and ammonium (\(\text{NH}_4^+\)) are the two main inorganic N forms in the soils. \(\text{NH}_4^+\) 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 \(\text{NH}_4^+\), 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 \(\text{NH}_4^+\) 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 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 3SS 4x 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 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 \(\text{NH}_4^+\) as the N source. When grown in N-sufficient medium containing 10 \(\mu\text{M} \text{NH}_4^+\), 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 \(\mu\text{M} \text{NH}_4^+\), 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 con-
tained 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, 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, OsAMGAT1-D1, OsGO-
GAT1-D1 and double activation mutants were 119.5, 126.8,
115.6, 117.1 and 120.7% of that of WT, respectively (Fig-
ure 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 mon-
tored 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 OsGO-
GAT2 (Figure S2e,f), thus suggesting that these two genes
played minor roles in the better performance of the activa-
tion 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

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**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 sig-
nificant differences between genotypes as determined using Student’s $t$ tests. *$P < 0.05$. © 2020 The Authors.
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labeling using 7-day-old seedlings grown in hydroponic culture (Figure 2). Shoots and roots were harvested 12, 24, 36 and 48 h after 15N treatment. 15N 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/D/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 15N, total N and ammonium in the shoots of WT and mutant seedlings (Figure 2c,d). The expression levels of OsAMT1;1 and OsAMT1;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 15NH4+ (Figure 2f,g).

To confirm 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 15N tracing assay. Plants were labelled with 15NH4Cl-containing Yoshida solution until the seventh leaf stage from germination (time T0). Labeled roots and leaves were harvested at T0 from five plants per genotype. The remaining five plants were transferred to 15N free solution and were grown until the tenth leaf stage over 3 weeks (time T1 is the end of chase period). At T1, roots, old leaves previously labeled at T0 (from the first to seventh leaves) and leaves newly emerged after T0 (eight to tenth leaves) were harvested separately for 15N analysis. At T0, there were no significant differences in the 15N distribution patterns between WT and mutant plants (Figure 3a). At T1, 15N partition patterns were similar in WT and OsAMT1;2-D2 plants...
leaves were harvested from five plants. The remaining plants were transferred to Yoshida solution containing $^{14}$NH$_4$Cl instead of $^{15}$NH$_4$Cl, and grown over 3 weeks until the tenth leaf stage (time T1; 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 T0).
(b) Partitioning of $^{15}$Na at T1 in roots, old leaves, and newly emerged three leaves during the chase period.

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 5b,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$_4$Cl containing Yoshida solution until the seventh leaf stage (time T0). Labeled roots and leaves were harvested from five plants. The remaining plants were transferred to Yoshida solution containing $^{15}$NH$_4$Cl instead of $^{15}$NH$_4$Cl, and grown over 3 weeks until the tenth leaf stage (time T1; 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 T0).
(b) Partitioning of $^{15}$N at T1 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.

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those of WT (Figures 5a and S6a,b). Plant height, above-ground 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 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.

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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 an 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...
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-derespressible (Sonoda et al., 2003). There were two contrasting results of overexpression 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 increases 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 OsAMT1;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. Accordingly, 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 (Mifflin 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 overexpression plants in the greenhouse or under field conditions (Brauer et al., 2011). The
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 1a–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.
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 NH₄Cl as the only N source, and 5 mM KCl instead of 5 mM KNO₃. For nitrogen sufficiency, 10 mM NH₄Cl added to modified MS medium as N source, and KCl was substituted for KNO₃. 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, above-ground 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 ΔΔCt 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 MICRO 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) sulfoisalicylic acid and quantified as described previously (Rosen, 1957).

Measurement of NH₄⁺ and GS activity

For free NH₄⁺ 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 NH₄⁺ in the supernatant was determined using Berthelot color reaction method (Rhine et al., 1998). Absorbance at 635 nm was determined and NH₄⁺ contents were calculated from the standard curve of (NH₄)2SO₄. 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 MgCl₂, 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), MgCl₂ (150 mM), EDTA (30 mM), NH₂OH (6 mM), ATP (60 mM) and Tris-HCl (50 mM). After 30 min of incubation, the reaction was stopped adding a solution containing FeCl₃, 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.

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

For ¹⁵N 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 NH₄NO₃ was replaced by 2.88 mM NH₄Cl. Next, seedlings were treated with ¹⁵N-labeled NH₄Cl (10% atom ¹⁵NH₄Cl; 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 ¹⁵N remobilization efficiency, seeds were germinated and cultured in the Yoshida solution containing ¹⁵N-labeled 2.88 mM NH₄Cl (10% atom ¹⁵NH₄Cl; Sigma-Aldrich) until the emergence of the seventh leaves (time T₀). At T₀, 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 T₁; chase period). At T₁, 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 ¹⁵N 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. Growth of WT and activation tagging mutants grown under N sufficiency or N limitation.

Figure S5. Leaf senescence phenotype of WT and mutants.

Figure S6. Expression analysis of OsGS2 and OsGOGAT2.
Double activation of OsAMT1.2 and OsGOGAT1

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