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Letter to the editor

Genetic manipulations of *TaARE1* boost nitrogen utilization and grain yield in wheat

Nitrogen is a key element essential for plant growth and crop production, and the improvement of the nitrogen use efficiency (NUE) of crops largely contributes to yield production. The improvement of NUE is a major challenge in agriculture, not only for reducing the planting cost of crops, but also for that of environmental pollution caused by the excessive application of nitrogen fertilizers (Bobbink et al., 2010; Ladha et al., 2016). Nitrogen utilization in nonlegume plants involves complex processes, mainly including absorption, transport, assimilation, and reutilization. Ammonium and nitrate, the two most abundant inorganic nitrogen in soil, are the primary nitrogen sources available for plants (Xu et al., 2012). Ammonium directly absorbed by ammonium transporter or generated from nitrate reduction is assimilated into amino acids via the glutamine synthase/glutamine: 2-oxoglutarate aminotransferase (GS/GOGAT) cycle. In rice, weak mutant alleles of the ferredoxin-dependent *GOGAT* (*Fd-GOGAT*; also known as *ABNORMAL CYTOKININ RESPONSE1* or *ABC1*) gene cause severe abnormalities associated with nitrogen deficiency, whereas null mutations are seedling lethal (Yang et al., 2016). Notably, the nitrogen-deficient phenotype of the *abc1* weak allele is partially suppressed by an *abc1 repressor1* (*are1*) mutation. Further analysis shows that *ARE1*, encoding an unknown function protein localized in the chloroplast, acts as a negative regulator of NUE (Wang et al., 2018). Mutations in *ARE1* cause significantly delayed senescence, enhanced NUE, and increased grain yield under nitrogen-limiting conditions (Wang et al., 2018, 2021).

Because *ARE1* is a highly conserved gene in the plant kingdom (Wang et al., 2018; Fig. S1 and S1B), we reasoned that the *ARE1*-like genes in other crops may also represent potential targets for breeding high-yield cultivar with improved nitrogen utilization. To test this possibility, we functionally analyzed *TaARE1*, the rice *ARE1* ortholog in wheat, focusing on NUE and grain yield. Common wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD) is a major staple crop worldwide, along with both planting area and yield production ranking first among crops. In the wheat genome, a single locus of rice *ARE1*-like gene was identified, located in three wheat subgenomes (A, B, and D), designated as *TaARE1-A*, *TaARE1-B,* and *TaARE1-D* genes, respectively (Fig. S2A). Although rice *ARE1* and wheat *TaARE1* are more than 80% identical at both nucleotide and protein levels, the identity of the three *TaARE1* homoeoalleles were ~99% (Fig. S1A and S1B). We generated wheat *taare1* mutations using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing system (Wang et al., 2014; Li et al., 2019; Liu et al., 2020).

To simultaneously introduce mutations in all three *TaARE1* copies, we adopted the CRISPR-Cas9 system targeting at a highly conserved region in exon 1 of *TaARE1* in the winter wheat variety Kenong199 (KN199) genetic background (Fig. S2A). The initial transformants were selfing, and the resulting progenies were analyzed by DNA sequencing in the targeting regions. No mutations were detected in the potential off-target regions (Table S1). Four homozygous null *taare1* mutants were obtained, including two mutant alleles in *TaARE1-A* (*are1-a1* and *are1-a2*; aaBBDD), one mutant in *TaARE1-A* and *TaARE1-B* (*are1-a3b1*; aabbDD), and one mutant in *TaARE1-B* and *TaARE1-D* (*are1-b2d1*; AAbbdd; Figs. 1A and S2B). The natures of the mutations included deletion, insertion, and deletion/insertion, all of which caused frame-shift mutations, most likely being null alleles (Figs. 1A and S2B). These *taare1* mutant plants were selfed for four times, and the resulting fourth generation plants and their progenies were used in all experiments hereafter unless specified otherwise.

When grown under field conditions, the *taare1* mutant plants showed no substantial difference compared with wild-type KN199 plants at the vegetable growth stages. However, these *taare1* mutants exhibited a slightly increased plant height and a delayed senescence phenotype at the mature stage (Fig. S2C), similar to that observed in the rice *are1* mutant plants (Wang et al., 2018). We noticed that the *are1-a3b1* mutant showed the most apparent stay-green phenotype in spikes and leaves, followed by *are1-a1* and *are1-a2*. Although the stay-green phenotype in leaves of *are1-b2d1* is relatively weak, the spikes showed a phenotype similar to other mutant plants (Fig. S2C). Notably, no obvious difference in the heading date was observed between KN199 and the *taare1* mutant plants. Under nitrogen-limiting conditions, an increasing root-to-shoot ratio represents an efficient strategy for plants to adapt to the deficient nitrogen environment (Andrews et al., 2013). Nitrogen deficiency significantly inhibited shoot growth but promoted root growth in both KN199 and the *taare1* mutant plants (Fig. S3A). The *taare1* mutants had a greater biomass of shoots and roots compared with KN199 under sufficient nitrogen conditions (Fig. S3B and S3C). Consequently, the root-to-shoot ratios of all four *taare1* mutants were substantially higher than that of KN199 seedlings under nitrogen-deficient conditions (Fig. 1B), suggesting that *taare1* mutations enhance tolerance to nitrogen deficiency and *TaARE1* modulates nitrogen utilization in wheat.

To further test the regulatory role of *TaARE1* in nitrogen utilization, we examined the responses of the *TaARE1* homoeoalleles to nitrogen availability. Nitrogen deficiency rapidly induced the expression of *TaARE1-A* and *TaARE1-B* in the shoots, peaked after nitrogen deprivation for 1 h. However, no response of *TaARE1-D* to nitrogen deprivation was detected (Fig. 1C). These results suggest that *TaARE1-A* and *TaARE1-B* are likely two main homoeoalleles involved in the nitrogen utilization in wheat.

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Given that mutations in *TaARE1* confer the tolerance to low nitrogen, we then performed a field trial to evaluate its function in modulating nitrogen utilization and productivity. The *taare1-a3b1* mutation showed the most profound effect on the stay-green phenotype, resulting in a significantly delayed milk-filling process and the loss of grain yield. Therefore, the *taare1-a1*, *taare1-a2*, and *taare1-b2d1* mutants were analyzed in subsequent studies (Fig. 1D). Under both high nitrogen (HN; 375 kg/ha urea) and low nitrogen (LN; 150 kg/ha urea) growth conditions, all three *taare1* mutants showed an increase in the plant height (Fig. S3D) and a delayed senescence phenotype (Fig. S3E). Compared with that of KN199, the tiller number remained nearly unaltered in *taare1-a1* and *taare1-a2* but significantly increased in *taare1-b2d1* (Fig. 1E). However, the grain number per main spike was increased for all three mutants under both HN and LN conditions (Fig. 1F). The grain weight was decreased in all three mutants at varying degrees under HN condition, owing to the incomplete filling, while increased in *are1-a1* and *are1-a2* under LN conditions (Fig. 1G). Nevertheless, grain yield per plant of all three mutants was increased, particularly under LN conditions (Fig. 1H), accompanying with the increased NUE (Fig. 1I). Notably, the introduction of mutations in *TaARE1* locus substantially increased the grain yield by ~6%−10% under HN conditions and ~3%−8% under LN conditions in the tested plots (Table S2). Taken together, these results show that mutations in *TaARE1-A* and *TaARE1-B*/*TaARE1-D* cause an increased NUE and boost grain yield under nitrogen-limiting conditions, providing a potential strategy for the genetic improvement of grain production in wheat.

Rice *ARE1* is characterized as a negative regulator of NUE and grain yield (Wang et al., 2018, 2021). In this study, we find that *TaARE1* plays a similar role in wheat by negatively regulating NUE and grain yield. Notably, because the genome structure of the allohexaploid wheat is more complicated than that of the diploid rice, copy number variations may play a more important role in determining the genetic function of a gene in wheat. Consistent with this notion, the expression of three *TaARE1* homoeoalleles shows distinctive responses to nitrogen availability. In agreement with the observation that the expression of *TaARE1-A* and *TaARE1-B*, but not *TaARE1-D*, is responsive to nitrogen, plants carrying the null

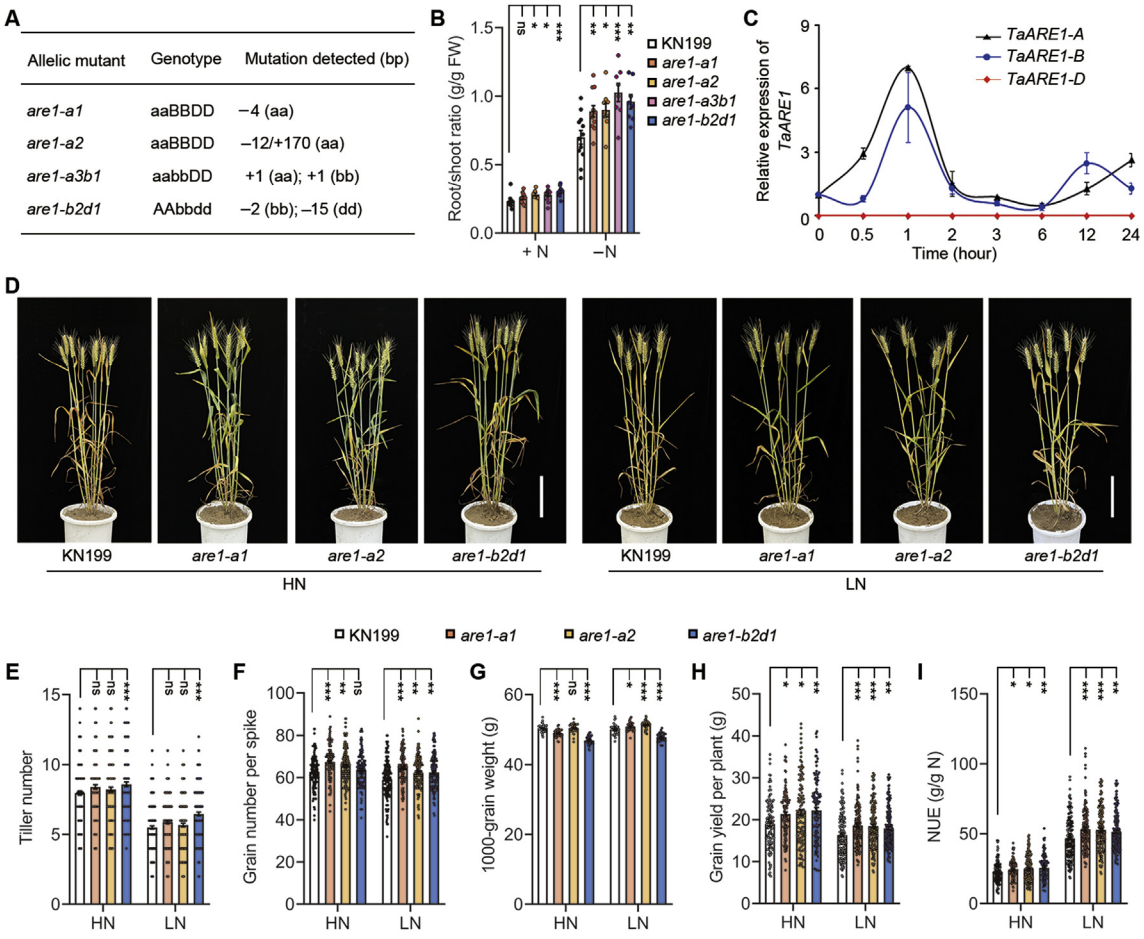

Fig. 1. Increased nitrogen utilization and grain yield in *TaARE1*-editing wheat plants. **A**: Four *taare1* mutant alleles generated by the CRISPR-Cas9 system, with "−" and "+" indicating deletion and insertion of the given number of nucleotides, respectively. **B**: Quantitative analysis of the root-to-shoot ratio of the indicated seedlings hydroponically cultured in a nitrogen-containing (+N) or nitrogen-free (−N) solution. Data presented are mean ± SEM by analyzing at least eight replicates. **C**: Analysis of nitrogen responses of three *TaARE1* homoeoalleles. Two-week-old wild-type KN199 seedlings hydroponically cultured in a nitrogen-containing solution were transferred to a nitrogen-free solution (time 0), and then cultured for the indicated time. Total RNA prepared from shoots was used for the qRT-PCR analysis. Data are means ± SD obtained from three technical replicates and each replicate consists of at least six seedlings. **D**: The phenotype of KN199 and three *taare1* mutant plants at the mature stage grown under HN (375 kg/ha urea) or LN (150 kg/ha urea) conditions. The images are taken for individual plants randomly selected from field-grown population and then placed in pots. **E**–**I**: Quantitative analysis of the tiller number per plant (**E**), grain number per main spike (**F**), 1000-grain weight (**G**), grain yield per plant (**H**), and nitrogen utilization efficiency (**I**) of plants shown in (**D**). Plant NUE was determined by the ratio of actual grain yield to applied fertilizer nitrogen per plant. All data are means ± SEM obtained from three biological replicates, and each replicate consists of at least 40 plants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). Scale bars, 15 cm (**D**). FW, fresh weight; HN, high nitrogen; LN, low nitrogen; NUE, nitrogen utilization efficiency; ns, not significant; SEM, standard error of mean; SD, standard deviation.

taare1-a3b1 mutations in *TaARE1-A* and *TaARE1-B* show a remarkably stronger phenotype of the delayed senescence than that of *are1-a1*, *are1-a2*, and *are1-b2d1*. Moreover, although the coding sequences of three *TaARE1* homoeoalleles are highly conserved (Fig. S1A), their putative promoter sequences (2 Kb upstream from the putative transcription start; Fig. S4), the locations, and length of their introns are significantly divergent (Fig. S2A), with a more apparent divergence between subgenomes A/B and D. These observations raise the possibility that their differential expression patterns in response to nitrogen may be partly attributed by regulatory elements embedded in the promoters and introns. In this regard, considering that the rice *ARE1* expression is subjected to the regulation by genetic variations in the promoter and introns (Wang et al., 2018, 2021), it will be of great interest to explore if a similar mechanism is also operated in wheat. Such potential regulatory elements or genetic variations will be valuable resources for the breeding of high NUE varieties in wheat. Moreover, pyramiding of these potentially elite alleles with other NUE-promoting genes or combined with genome editing will be a useful approach in breeding as demonstrated in rice (Xu et al., 2019; Biswas et al., 2020; Wang et al., 2021). Finally, the demonstration of the conserved *ARE1* and *TaARE1* genes in regulating NUE and productivity in rice and wheat (Wang et al., 2018; Zhang et al., 2021) implies the potentials for the genetic improvement of other crops as well.

Conflict of interest

The authors declare they have no conflict of interest.

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Supplementary data

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