Ectopic expression of fungal EcGDH improves nitrogen assimilation and grain yield in rice

Summary  NADP(H)-dependent glutamate dehydrogenases (GDH) in lower organisms have stronger ammonium affinity than those in higher plants. Here we report that transgenic rice overexpressing the EcGDH from Eurotium cheralieri exhibited significantly enhanced aminating activities. Hydroponic and field tests showed that nitrogen assimilation efficiency and grain yields were markedly increased in these transgenic plants, especially at the low nitrogen conditions. These results suggest that EcGDH may have potential to be used to improve nitrogen assimilation and grain yield in rice.

Glutamate dehydrogenase (GDH), which exists widely in the biosphere, catalyzes the reversible reaction for the reductive amination of 2-oxoglutarate (2-OG) to produce glutamate in the presence of the cofactor NAD(P)H (Wootton 1983). It has been reported that two pathways are involved in the primary assimilation of ammonium in higher plants, one of which is glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway, and the other is GDH pathway (Skopelitis et al. 2007). Owing to the lower affinity of GDHs for ammonium in higher plants, nitrogen is thus mainly assimilated by the GS/GOGAT pathway. However, GDHs originating from lower organisms such as fungi can more efficiently assimilate nitrogen due to their higher affinity for ammonium. Hence, some GDHs from lower organisms were introduced into crops (Abiko et al. 2010; Du et al. 2014; Zhou et al. 2015a), where these heterologous GDHs can only improve the nitrogen assimilation but not necessarily production in crops (Du et al. 2014; Zhou et al. 2014). Thus, it prompts us to isolate and identify new GDHs from lower organisms, which can improve both nitrogen assimilation and production in crops. Here we cloned an NADP(H)-GDH gene (EcGDH) from a fungus Eurotium cheralieri and introduced it into rice (Oryza sativa cv. Kitaake). The results of hydroponic and field tests demonstrate that the heterologous EcGDH significantly improves nitrogen assimilation and grain yield in transgenic rice under low-nitrogen field conditions.

To analyze the enzymatic property of EcGDH, both the recombinant protein His-Trigger Factor (TF)-EcGDH and His-TF-OsGDH were expressed in E. coli BL21 (DE3) and purified (Figure 1A–C). Enzyme activity assay performed in vitro demonstrated that the NADP(H)-GDH activities, especially the aminating activity, of the His-TF-EcGDH were significantly higher than those of His-TF-OsGDH (Figure 1D), and the Km value of EcGDH for ammonium is 2.34 ± 0.21 mmol/L (Table S1), which is significantly lower than that of OsGDH reported (12.43 ± 1.84 mmol/L; Zhou et al. 2014). These results indicate that EcGDH with a strong affinity for ammonium is able to assimilate NH4 + even at very low concentration. In addition, we found that the EcGDH protein was located primarily in the cytoplasm (Figure 1E), which is similar to another ammonium assimilation-related enzyme, GS protein (Obara et al. 2000). We suspect that the co-localization of EcGDH with GS can synergistically improve ammonium assimilation in the cytoplasm.

To assess the impact of EcGDH on nitrogen assimilation in rice, EcGDH was firstly codon-optimized according to the rice codon bias (Figure S1), and then ectopically expressed in rice (Figures 1G, S2). The transgenic plants carrying EcGDH, expressed under the control of an ubiquitin promoter (Figure S2A), exhibited significantly increased aminating activity when grown under normal conditions as compared with non-transgenic control plants, while their deaminating activities were only slightly enhanced (Figure 1H). Hydroponic test showed that, compared with control seedlings, the growth of transgenic seedlings were only slightly inhibited (Figure 1F, I, J), although no effect on total dry weights was observed (Figure 1K). The number of roots in these transgenic plants was increased to some extent (Figure 1L). Furthermore, the contents of
Figure 1. Characterization of EcGDH and phenotypic analysis of the transgenic rice seedlings

(A) His-TF-EcGDH purified from *Escherichia coli*. M protein marker; 1, purified His-TF-EcGDH; 2, before purified His-TF-EcGDH; 3, purified His-TF. (B) His-TF-OsGDH purified from *E. coli*. M protein marker; 1, purified His-TF-OsGDH; 2, before purified His-TF-OsGDH. (C) His-TF-EcGDH identification by western blot with an anti-His antibody. (D) Enzyme activity assay of His-TF-EcGDH and His-TF-OsGDH. (E) Investigation of subcellular localization of EcGDH protein in *Arabidopsis* protoplast under an inverted fluorescence microscope. YFP yellow fluorescent protein. (F) Phenotypes of transgenic and control seedlings grown at different nitrogen concentrations (50 and 500 μM NH₄NO₃) for 20 d. Scale bars = 5 cm. (G) Expression analysis of EcGDH in rice by western blot. Ponceau was used as a loading control. (H) NADP(H)-GDH activities in EcGDH transgenic and control seedlings grown under normal conditions (half MS medium). (I–O) Statistics of the shoot lengths (I), root lengths (J), dry weights (K), root numbers (L), relative chlorophyll contents (M), total amino acids contents (N), and nitrogen contents (O) in the transgenic and control seedlings. OE-12, pUbi::EcGDH-12; OE-13, pUbi::EcGDH-13. Data are presented as average values ± SD (n = 3) in (B–H). P ≤ 0.05 (*); P ≤ 0.01 (**). Student's t-test.
parameters were evaluated at harvest time (Table 1). The nitrogen level of the paddy field where early rice was grown without application of any nitrogen fertilizer is defined as 0 kg/ha (the actual fertility was 0.65 g N/kg soil). Data are represented mean values ± SEM (n = 20). *P ≤ 0.05 (*); **P ≤ 0.01 (**), student’s t-test.

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chlorophyll, amino acids, and nitrogen in *pUbi::EcGDH* transgenic seedlings were significantly increased as compared with control seedlings (Figure 1M–O). These results indicate that overexpression of *EcGDH* can markedly improve nitrogen assimilation in rice at the seedling stage.

To evaluate the effects of *EcGDH* on agronomic traits of rice, the transgenic and control plants were grown at different nitrogen fertility, and the yield parameters were evaluated at harvest time (Table 1). All 1,000-grain weight, panicle number/hill, and grain yield/plant of *pUbi::EcGDH* transgenic lines were significantly higher than those of control plants at lower nitrogen fertility (0 and 37.5 kg N/ha), while no statistically significant differences were observed at higher nitrogen fertility (112.5 kg N/ha). These results demonstrate that ectopic expression of *EcGDH* can considerably increase grain yield under low nitrogen conditions.

So far, although several fungal GDH genes have been introduced into rice cultivars (Abiko et al. 2010; Du et al. 2014; Zhou et al. 2014; Zhou et al. 2015a, 2015b), only *CeGDH* from *Cylindrocarpon ehrenbergii* can increase the grain yield of transgenic food rice under low nitrogen conditions (Zhou et al. 2015a). Most recently, Zhang et al. (2016) reported that the *gdhA* from *Aspergillus niger* can improve the grain yield of a forage rice under low nitrogen conditions, while producing a similar effect only under high nitrogen conditions for the transgenic food rice (Abiko et al. 2010). More interestingly, only one of two *CeGDH* transgenic lines but both *EcGDH* transgenic lines investigated exhibited a significant increase of grain yields at lower nitrogen levels (Zhou et al. 2015a; Table 1). Therefore, we assume that *EcGDH* is a better fungal GDH gene than *CeGDH* with the potential for improving rice production at low nitrogen fertility.

In conclusion, ectopic expression of *EcGDH* from *Eurotium heralieri* can enhance the nitrogen uptake efficiency and increase grain yield in rice at the low nitrogen fertility.

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AUTHOR CONTRIBUTIONS

D. T., Y. P. and J. L. performed most of the research and J. L. drafted the manuscript. C. D. and D. W. carried out the kinetic analysis, Y. Y. and C. L. performed field tests. L.Y., X. Z., and X.L. carried out hydroponic tests. L. C. and X. L. designed the experiment, supervised the study and revised the manuscript.

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Figure S1. Comparison of original and modified sequences of EcGDH

Figure S2. Cloning of EcGDH and identification of the transgenic rice plants

Table S1. Kinetic property of purified EcGDH protein in vitro

Table S2. Primers used in this study
EcGDH improves nitrogen assimilation and grain yield

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