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Metabolic engineering of theanine biosynthesis in rice endosperm to develop biofortified theaRice for benefiting human health

Dear Editor,

Theanine (N-ethyl-γ-L-glutamine) is a characteristic amino acid primarily found in tea leaves (*Camellia sinensis*). It is derived from pyruvate through three enzymatic steps involving alanine aminotransferase (AlaAT), alanine decarboxylase (AlaDC), and theanine synthetase (TS) (She et al., 2022; Luo and He, 2025). Theanine has attracted widespread attention due to its taste-enhancing properties and potential health benefits, including relaxation and cognitive enhancement (Li et al., 2022), however, its limited natural production, coupled with increasing demand, has seriously restricted its broad application. Recent studies have demonstrated that *Nicotiana benthamiana* leaves and microbial systems can be engineered to generate high yields of theanine (Benninghaus et al., 2021; Zhu et al., 2021). Nevertheless, unstable gene expression, high production costs, and the risk of environmental pollution pose serious challenges for scaling this process. In contrast, crops—especially rice (*Oryza sativa* L.) endosperm—can serve as ideal bioreactors to produce nutrients and bioactive metabolites such as oil and CoQ_{10} (Liu et al., 2024; Xu et al., 2025) in a process also known as plant molecular farming (Zhu et al., 2022). Despite these advancements, the biological effects of most novel biofortified crops have yet to be explored. These crops possess complex components that may display synergistic or antagonistic effects, potentially generating novel physiological responses (Supplemental Figure 1). Here, we aimed to enhance both the stability and accumulation of theanine in rice endosperm, and to evaluate the physiological effects of this biofortified rice—an approach that has substantial potential for biofortification and biomanufacturing applications.

To biosynthesize theanine in rice endosperm, the coding sequences of two critical enzymes from *Camellia sinensis L.*, CsAlaDC and CsTS, were codon-optimized for expression in rice. The recombinant forms *rAlaDC* and *rTS* (Supplemental Table 1) were linked via the F2A peptide coding sequence isolated from the foot-and-mouth disease virus (FMDV) to form a single open reading frame. The *rAlaDC* and *rAlaDC-F2A-rTS* units were placed under the control of the endosperm-specific P_{GluB4} promoter and cloned into a Cre/*loxP*-mediated marker auto-elimination vector, generating constructs ***D*** and ***DT***, respectively (Figure 1A). A feedback-insensitive OsAlaAT enzyme from *O. sativa* L. was also placed under the control of the P_{GluB1} promoter to enhance alanine biosynthesis, a rate-limiting step in theanine production, and this expression cassette was then added to ***DT***, generating the ***DTA*** construct (Figure 1A). All constructs were transformed into the *indica* rice cultivar NanGuiZhan (NGZ) via *Agrobacterium*-mediated transformation, yielding 23, 28, and 26 independent transgenic (T_0) lines, respectively. The insertions and expression of transgenes were then further confirmed (Supplemental Figures 2A and 2B).

To determine metabolite composition and content, high-performance liquid chromatography was used to quantify key metabolites in NGZ and transgenic grains (Supplemental Figures 3A–3D). Ethylamine, the direct precursor of theanine biosynthesis, was only detected in ***DTA*** lines at a concentration of 8.70 μg g^{-1}. Theanine production was significantly higher in ***DTA*** grains (∼100 μg g^{-1}) compared to ***DT*** grains (0.90 μg g^{-1}) (Figures 1B and 1C). Co-expression of *OsAlaAT* and the *rAlaDC-F2A-rTS* unit led to a substantial increase in alanine accumulation in ***DTA*** lines compared to NGZ and ***DT***. Interestingly, GABA content in ***DT*** and ***DTA*** lines was 4.7–15.7 times higher than that in NGZ (Supplemental Table 2), possibly due to conversion from glutamine to maintain glutamate–glutamine homeostasis. These results suggest that *OsAlaAT* enhances theanine biosynthesis by promoting alanine accumulation, thereby increasing overall metabolite flux toward theanine production.

All transgenic plants exhibited normal growth and yields comparable to NGZ. However, the endosperm of ***D*** plants exhibited increased chalkiness (Supplemental Figures 4A–4C), suggesting potential disruptions in starch synthesis. We performed RNA sequencing (RNA-seq) analyses of NGZ and the transgenic lines and identified 393 common differentially expressed genes (DEGs) between the transgenic lines and NGZ ($P < 0.05$, absolute $\log_2$fold change ≥ 1) (Supplemental Figures S5A and S5B). Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed that these DEGs were significantly enriched in energy metabolism and carbon-cycle-related pathways (Supplemental Figure S5C). Selected DEGs were further analyzed by real time qPCR. Compared to NGZ, genes involved in disaccharide and pyruvate biosynthesis (e.g., *OsAmy3D* and *Osβ-GC)* exhibited an overall upward trend in transgenic plants (Figure 1D), indicating enhanced carbon assimilation and glycolysis. Components of the glutamate–glutamine metabolism module, including *OsGS1;3*, *OsGOGAT1*, and *OsGAD1*, were also activated in transgenic lines, consistent with the elevated glutamine and GABA levels observed in ***DT*** and ***DTA*** grains (Figure 1D; Supplemental Table 2).

A key advantage of biofortified crops is their potential to provide functional metabolites through oral ingestion. Given that ***DTA*** grains contained the highest levels of bioactive compounds such as theanine and GABA, this novel germplasm was designated as theanine rice (theaRice), and the homozygous marker-free theaRice (Supplemental Figures S6A–S6C) was utilized in animal feeding experiments. To evaluate the effects of theaRice

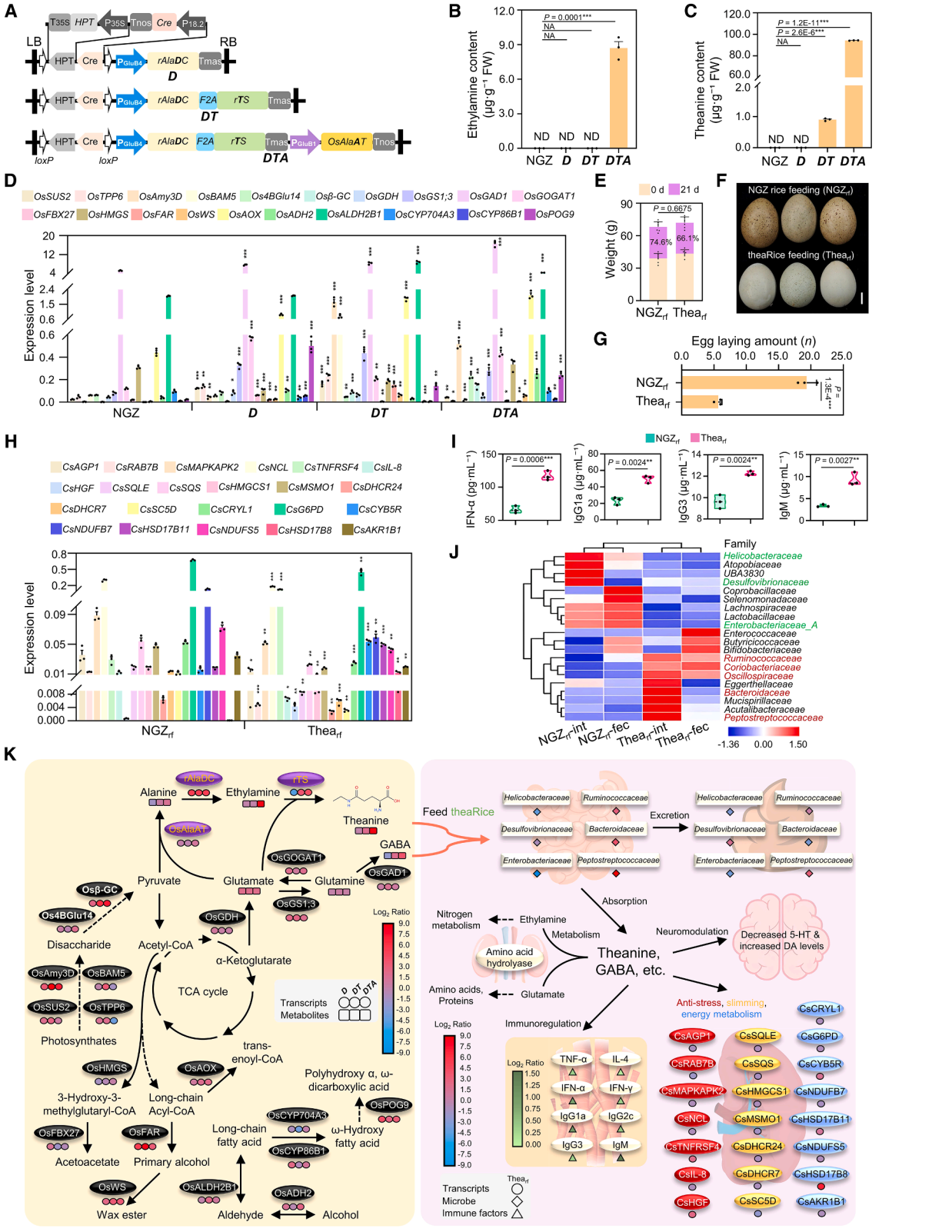

Figure 1. Characterization of engineered theanine rice (theaRice) and its *in vivo* efficacy.

(A) Schematic diagram of the construct for theanine biosynthesis in rice endosperm.

(B and **C)** Quantification of ethylamine **(B)** and theanine **(C)** extracted from the endosperm of NGZ and transgenic lines. Data are presented as means ± SE (n = 3), analyzed using Student's t-test. FW, fresh weight; ND, not detectable; NA, not available.

(legend continued on next page)

consumption on a living system, 1-month-old blue-breasted quails (*Synoicus chinensis*) were used as an animal model. After 3 weeks of feeding, quails in the theaRice-fed ($Thea_{rf}$) group exhibited a slight reduction in weight gain compared to the NGZ rice-fed (NGZ_{rf}) group (66.1% vs. 74.6%, on average) (Figure 1E). Additionally, eggs from the $Thea_{rf}$ group were lighter in color, and egg production was significantly reduced compared to the NGZ_{rf} group (5.7 vs. 19.3 eggs on average during the theaRice feeding period) (Figures 1F and 1G).

RNA-seq analyses of spleen samples from $Thea_{rf}$ and NGZ_{rf} groups identified a total of 942 upregulated and 724 downregulated DEGs ($P < 0.05$, absolute $\log_2$fold change ≥ 1) (Supplemental Figures 7A–7C). Gene Ontology enrichment analysis revealed significant enrichment of DEGs associated with sterol biosynthetic processes and oxidoreductase activity (Supplemental Figures 8A and 8B). Specifically, the transcription of several rate-limiting enzymes involved in cholesterol biosynthesis—*CsSQS*, *CsHMGCS1*, and *CsSC5D*—and energy metabolism—*CsG6PD*, *CsNDUFB7*, and *CsAKR1B1*—was downregulated in the $Thea_{rf}$ group (Figure 1H). In contrast, *CsHSD17B8* and *CsHSD17B11*, which encode hydroxysteroid dehydrogenases that convert high-activity estradiol into lower-activity estradiol and estriol (Ohno et al., 2008), respectively, were significantly upregulated by 2.7- to 86.5-fold in the $Thea_{rf}$ group (Figure 1H). These results suggest that theaRice consumption may suppress sterol accumulation, reduce energy expenditure, and attenuate estrogenic activity.

Furthermore, several DEGs, including *CsAGP1*, *CsIL-8*, and *CsTNFRSF4*, were associated with inflammatory and interleukin signaling pathways and immune response processes (Figure 1H; Supplemental Figures 8A and 8B), suggesting that theaRice consumption may enhance stress resistance and immune function. Indeed, blue-breasted quails in the $Thea_{rf}$ group exhibited reduced activity levels and fewer signs of restlessness and conflict behaviors than the NGZ_{rf} group (data not shown). To further investigate the immunomodulatory effects of theaRice, enzyme-linked immunosorbent assays were used to quantify cytokine levels in serum samples from the NGZ_{rf} and $Thea_{rf}$ groups. Significant increases (7.3%–175.9%) were observed in the protein levels of several cytokines in group $Thea_{rf\ \text{compared to group}\ NGZ_{rf}}$, including tumor necrosis factor alpha, interleukin-4, interferon (IFN)-α, IFN-γ, IgG1a, IgG2c, IgG3, and IgM. Notably, IFN-α and IgM levels were 1.77-fold ($P = 0.0006$) and 2.76-fold ($P = 0.0027$) higher, respectively, in group $Thea_{rf}$ than in group NGZ_{rf} (Figure 1I; Supplemental Figure 9). These findings suggest that the consumption of theaRice enhances cytokine production and improves immune function.

The intestinal microbiota plays a crucial role in animal health and disease (Thursby and Juge, 2017). To assess the impact of theaRice on gut microbiota composition, 16S rRNA gene sequencing was performed on blue-breasted quails. A significantly higher abundance of gut microbiota was observed in group $Thea_{rf}$ than in group NGZ_{rf}, although no significant differences in microbial diversity were detected in fecal samples (Supplemental Figures 10A and 10B), suggesting that theaRice consumption enhances the selective retention of specific microbial communities in the intestine. Further analysis of microbial community composition revealed a reduced abundance of pathogenic bacterial families, including *Helicobacteraceae*, *Desulfovibrionaceae*, and *Enterobacteriaceae* (highlighted in green in Figure 1J), in both the intestine and feces of group $Thea_{rf}$. Conversely, beneficial bacterial families such as *Ruminococcaceae*, *Coriobacteriaceae*, *Oscillospiraceae*, *Bacteroidaceae*, and *Peptostreptococcaceae* (highlighted in red), which are crucial for maintaining intestinal health and promoting digestion, were enriched in group $Thea_{rf}$ (Figure 1J; Supplemental Table 3).

We integrated the theanine, carbohydrate, and fatty acid biosynthesis pathways into a transcriptional cascade and metabolic network, showing that metabolites and enzymes in these pathways are significantly upregulated (Figure 1K). Following theaRice consumption, its active metabolites were absorbed by intestinal cells and entered the bloodstream, where they contributed to immunoregulation, stress resistance, and gut health (Figure 1K). While other biofortified crops—such as high-oleic-acid rice and CoQ_{10} rice—also produce health-promoting metabolites (Liu et al., 2024; Xu et al., 2025), their commercialization has faced varied public concerns and inconsistent regulatory policies across countries. Boosting international collaboration to establish unified regulatory frameworks and standards will be critical to promoting the industrialization of genome-edited crops (e.g., CoQ_{10} rice) and genetically modified crops (e.g., high-oleic-acid rice and theaRice). Overall, our study presents a successful example of biofortified crop development with demonstrated health benefits.

DATA AND CODE AVAILABILITY

The RNA-seq data have been deposited in the China National Center for Bioinformation (https://www.cncb.ac.cn/) at the Beijing Institute of

(D) Expression analysis of selected endogenous differentially expressed genes (DEGs) in the grains of NGZ and homozygous T_3 lines. Data are shown as means ± SE ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). *OsActin1* was used as a reference gene.
(E) Average body weight of blue-breasted quails before (orange) and after (purple) 3 weeks of feeding with NGZ (NGZ_{rf}) or theanine rice ($Thea_{rf}$), with the weight gain rates. Data are shown as means ± SE ($n = 6$). D, days.
(F) Eggs laid by the NGZ_{rf} and $Thea_{rf}$ groups. Scale bar: 1 cm.
(G) Total number of eggs laid by the NGZ_{rf} and $Thea_{rf}$ groups. Data are shown as means ± SE ($n = 3$).
(H) Validation of select DEGs in spleen samples from the NGZ_{rf} and $Thea_{rf}$ groups. Data are shown as means ± SE ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). *CsActin1* was used as a reference gene.
(I) Concentrations of IFN-α, IgG1a, IgG3, and IgM in serum samples from the NGZ_{rf} and $Thea_{rf}$ groups. Data are shown as means ± SE ($n = 3$).
(J) Heatmap of the top 20 bacterial families enriched in intestinal (int) and fecal (fec) samples from the $Thea_{rf}$ group compared to the NGZ_{rf} group. Log_2 fold changes are shown, with enriched families in red and depleted families in blue. Relevant families are highlighted.
(K) Integrated transcriptional and metabolic network in theaRice and in animals fed with theaRice. Data were log-transformed (values from the NGZ or NGZ_{rf} groups were set to 0.0 as references). Black dotted lines indicate omitted metabolic steps.

Genomics, Chinese Academy of Sciences, under accession numbers PRJNA1227772 for rice endosperms and PRJNA1227911 for spleens of blue-breasted quails. The sequence data for rice (*O. sativa* L.) and blue-breasted quail (*S. chinensis*) genes reported in this study can be found in the Rice Genome Annotation Project (RGAP; http://rice.uga.edu/) and the China National Center for Bioinformation (https://www.cncb.ac.cn/), respectively. The gene accession numbers used in this study are listed in Supplemental Table 5. The authors declare that all data supporting the findings of this study are available within the manuscript and its supplemental information or are available from the corresponding authors upon reasonable request.

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AUTHOR CONTRIBUTIONS

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SUPPLEMENTAL INFORMATION

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