Letter to the Editor

Potential ‘accelerator’ and ‘brake’ regulation of theanine biosynthesis in tea plant (Camellia sinensis)

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Dear Editor,

Theanine, one of the most important tea quality components, confers the ‘umami’ taste and anti-stress benefits of tea infusion [1]. Theanine is a unique non-proteinaceous amino acid solely accumulating to a high level in tea plants, and is primarily synthesized in the roots from glutamate (Glu) and ethylamine (EA) by theanine synthetase (CsTSI) [1, 2]. The high EA availability was suggested to be why only tea plants can synthesize large amounts of theanine [3]. Our recent study further indicated that EA contents in the roots of various tea plant cultivars were highly correlated with the theanine contents in the roots [4]. EA is synthesized from alanine by alanine decarboxylase CsAlaDC [4, 5]. Further experiments suggested that the CsAlaDC and CsTSI work in tandem to determine theanine biosynthesis in tea plants [4]. Interestingly, the expression of CsTSI in the roots of different tea plant cultivars was not obviously correlated with the theanine contents; while the expression of CsAlaDC was shown to be highly correlated with the theanine contents in the roots [4]. EA is synthesized from alanine by alanine decarboxylase CsAlaDC [4, 5]. Further experiments suggested that the CsAlaDC and CsTSI work in tandem to determine theanine biosynthesis in tea plants [4]. Interestingly, the expression of CsTSI in the roots of different tea plant cultivars was not obviously correlated with the theanine contents; while the expression of CsAlaDC was shown to be highly correlated with the theanine contents in the roots [4]. These results indicated that the regulation of CsAlaDC expression is critical for adjusting the activity of theanine synthesis in tea plants. However, the mechanism underlying the regulation of CsAlaDC expression is largely unknown in tea plants. Uncovering the mechanism will be critical for improving theanine biosynthesis in tea plants.

In our previous study, using weighted genome-wide co-expression network assay (WGCNA), we identified 16 MYB transcription factors which were highly correlated with the expression of theanine pathway genes under various conditions [6]. Within these 16 genes encoding MYB transcription factors, TEA011475.1 and TEA003248.1 were up- and down-regulated, respectively, by nitrate, ammonium, and EA treatments [6], suggested these two genes might play important and divergent roles in regulating theanine biosynthesis. Consistently, further experiments showed that, within the 16 genes, the expression of TEA011475.1 and TEA003248.1 showed the highest positive and negative correlation with theanine contents in the roots, respectively, under 20 EA mM treatment for 6 h, 12 h, 1 d, 3 d, and 6 d [6] (Fig. 1A). We hypothesized that these two MYB transcription factors regulate the expression of CsAlaDC. To verify our hypothesis, we first performed yeast one-hybrid assay, and the result suggested that TEA011475.1 and TEA003248.1 bound to the promoter of CsAlaDC (Fig. 1B).

In our other previous study, we performed transcriptomic analyses on the roots of tea plants treated with 0 N, NO₃⁺-N (1.43 mM), EA-N (1.43 mM), NH₄⁺-N (1.43 mM), or NO₃⁺ plus NH₄⁺-N (1.43 mM) for 10 days [7]. The transcriptomic data was used to construct the gene co-expression network of genes encoding transcription factors and CsAlaDC, with CsAlaDC as the hub gene, to identify potential transcription factors regulating CsAlaDC transcription (Fig. 1C). These genes encoding transcription factors from the gene co-expression network were further narrowed down by selecting root-specifically expressed genes. In this way, 13 CsAlaDC-associated and root-specific genes encoding transcription factors were obtained. Interestingly, both TEA011475.1 and TEA003248.1 were also included in these 13 genes. Again, the expression of TEA011475.1 and TEA003248.1 was shown to be respectively induced and repressed by N, and highly correlated with the

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Figure 1. CsHHO3 and CsMYB40 play opposite regulatory roles in CsAlaDC expression to regulate theanine biosynthesis in tea plants. (A) Expression of 16 theanine-associated genes encoding transcription factors (TFs) under ethylamine (EA) treatment for 6, 12, 24, 72, and 144 h, and the correlation coefficients with the theanine contents, in the roots. (B) Yeast one-hybrid assay to test the binding of TEA011475.1 and TEA003248.1 to CsAlaDC promoter. (C) Bipartite network of TFs-CsAlaDC gene co-expression network. (D) Expression of 13 root-specific TF encoding genes under the treatment of 0 N, 1.43 mM NO₃⁻+N, 1.43 mM EA-N, 1.43 mM NH₄⁺-N, or 0.36 mM NO₃⁻+ plus 1.07 mM NH₄⁺-N (NO₃⁻+NH₄⁺-N) for 10 days, and the Pearson correlation coefficients (PCC-value) with the theanine contents, in the roots. (E) Phylogenetic analysis of TEA011475.1 and TEA003248.1 with their homologues in Arabidopsis thaliana. (F–H) Tissue-specific expression patterns of CsAlaDC, CsHHO3 and CsMYB40. The expression level was determined by real-time quantitative RT-PCR. First MV, major vein in the first leaf; third MV, major vein in the third leaf. MV was obtained by separated the major vein from the leaf. (I) Subcellular localization of CsHHO3 and CsMYB40. (J) Transactivition assay of full-length or truncations of CsHHO3 and CsMYB40 in yeast. (K) A schematic illustration of the effector and reporter constructs in transcriptional activity of the CsAlaDC promoter. (L) Regulation of the transient transcriptional activity of the CsAlaDC promoter by CsHHO3 and CsMYB40 in tobacco epidermal cell. (M) Schematic representation of the fragments used for transcription factor binding site screening. (N, O) EMSA assay of CsHHO3 (N) and CsMYB40 (O) to exam the binding to different regions of CsAlaDC promoter. Cold probe represents the putative binding motif without biotin labeling. (P) Theanine contents in the roots of tea plants under 0 N, 1 N, 10 N, and 20 N treatments. (Q) The relative expression of CsAlaDC, CsHHO3, and CsMYB40 under the four nitrogen treatments. (R) The proposed 'accelerator' and 'brake' regulation model of theanine biosynthesis in tea plants. The arrows and bar-ended lines represent promotion and inhibition, respectively. Thick and thin lines mean strong and weak effects, respectively.
theanine contents in the opposite manner (Fig. 1D). These results further suggested critical and different roles of TEA011475.1 and TEA003248.1 in CsAlaDC expression and theanine biosynthesis.

According to the results of phylogenetic analysis, we named TEA011475.1 and TEA003248.1 as CsHHO3 and CsMYB40, respectively, following the names of their homologues in model plant Arabidopsis (Fig. 1E). The tissue-specific expression showed that both CsHHO3 and CsMYB40 specifically expressed in the roots, as well as CsAlaDC (Fig. 1F–H). Consistent with the prediction that CsHHO3 and CsMYB40 are transcription factors, CsHHO3 and CsMYB40 both localized in the nucleus (Fig. 1I). The results of transcription activation assay suggested that CsHHO3 did not have transcription activation activity, while the predicted transcription activation domain of CsMYB40 had transcription activation activity, although the full length of CsMYB40 did not (Fig. 1J). This is consistent with the homologue of CsHHO3 and CsMYB40 in Arabidopsis being transcription repressor and activator, respectively [8, 9].

To test the regulatory role of CsHHO3 and CsMYB40 in CsAlaDC expression, we next conducted a trans-activation assay in tobacco leaves using CsAlaDC promoter-driven Luciferace (CsAlaDC pro:Luc) as a reporter (Fig. 1K). The results showed that when co-transformation 35S:CsHHO3 and CsAlaDC pro:Luc, the expression of CsHHO3 significantly repressed the activity of CsAlaDC promoter (Fig. 1L). In contrast, the expression of CsMYB40 significantly activated the activity of CsAlaDC promoter. Moreover, when co-expressed 35S:CsHHO3 and 35S:CsMYB40 (CsHHO3 + CsMYB40) with CsAlaDC pro:Luc, the activity of CsAlaDC promoter was not obviously different from the no-effector control (CsAlaDC pro:Luc). These results demonstrated that CsHHO3 represses, and CsMYB40 activates, the expression of CsAlaDC in planta.

To investigate whether CsHHO3 and CsMYB40 directly bind to the CsAlaDC promoter, we conducted an electrophoretic mobility shift assay (EMSA) with recombinant CsHHO3 and CsMYB40 proteins. The binding sites were first screened by testing the binding of CsHHO3 and CsMYB40 with the 12 fragments (200 bp each) of the promoter (Fig. 1M). Through this screening, we found CsHHO3 bound to the proximal region (−476 to −277) of CsAlaDC promoter, and CsMYB40 bound to three regions (−427 to −626, −727 to −926, and −1027 to −1226) (Fig. 1N, O). By looking for cis-regulatory elements recognized by MYB TFs, the CsMYB40 binding regions were further narrowed to −612 to −562, −884 to −839, and −1204 to −1159. These regions include the MYB-core consensus [C/T]NGTT[G/T] identified in protein-binding microarray [10]. These results indicated that CsHHO3 and CsMYB40 can directly bind to different regions of the CsAlaDC promoter.

To further investigate the regulatory roles of CsHHO3 and CsMYB40 in CsAlaDC expression in response to N levels, we grew tea plants in hydroponic solution with various concentrations of N (0 N, 1 N, 10 N, and 20 N; 1 N was 0.72 mM NO₃⁻-N plus NH₄⁺-N) for 18 days. Compared with the 0 N, 1 N, and 10 N significantly increased theanine contents in the roots (Fig. 1P); however, 20 N did not further increase the content compared with 10 N. The changes of expression of CsAlaDC in the roots showed a similar pattern as that of theanine contents (Fig. 1Q). Under the same conditions, the expression of CsHHO3 was gradually induced by increased concentrations of N, while that of CsMYB40 was repressed (Fig. 1Q). These results implied that CsHHO3, the potential repressor of CsAlaDC transcription, is induced by higher levels of N; however, CsMYB40, the potential activator of CsAlaDC transcription, is repressed by higher levels of N. Therefore, CsHHO3 and CsMYB40 likely work in tandem to maintain the expression level of CsAlaDC within a certain range in response to N levels, to keep the robustness of theanine biosynthesis. This is just like driving a vehicle on an expressway, hitting the accelerator when the vehicle slows down, and hitting the brake when the speed is too high, to keep the vehicle running at a high but safe speed (Fig. 1R).

In brief, we identified CsHHO3 and CsMYB40 as a repressor and an activator of CsAlaDC transcription, and their opposite expression patterns and regulatory roles likely maintain the robustness of ethylamine and theanine biosynthesis in response to nitrogen fluctuations. This study demonstrates a potential ‘accelerator’ and ‘brake’ regulation mode of theanine biosynthesis in tea plants (Fig. 1R). In future, we will further knock down and overexpress CsHHO3 and CsMYB40 to verify the regulation mode when the gene transformation system is well established in tea plants.

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Author contributions

Z.Z., X.W., J.G., Q.C., and L.L. conceived the study and designed the experiments; J.G., B.Z., Y.C., S.L., S.Q., and F.M. carried out the experiments; J.G., B.Z., Y.C., and X.W. wrote the manuscript. All authors reviewed and approved the final manuscript.

Data availability

Data are available upon request to the corresponding author.

Conflict of interest

The authors declare that they have no conflict of interest.
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