


## Brief Communication

# CsAlaDC and CsTSI work coordinately to determine theanine biosynthesis in tea plants (*Camellia sinensis* L.) and confer high levels of theanine accumulation in a non-tea plant

Biying Zhu<sup>1,†</sup>, Jiayi Guo<sup>1,†</sup>, Chunxia Dong<sup>1,†</sup>, Fang Li<sup>1,2</sup>, Siming Qiao<sup>1</sup>, Shijia Lin<sup>1</sup>, Tianyuan Yang<sup>1</sup>, Yingling Wu<sup>1</sup>, Shilai Bao<sup>3,4</sup>, William J. Lucas<sup>5</sup> and Zhaoliang Zhang<sup>1,\*</sup> 

<sup>1</sup>State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei, Anhui, China

<sup>2</sup>College of Horticulture, Nanjing Agricultural University, Nanjing, China

<sup>3</sup>State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China

<sup>4</sup>School of Life Sciences, University of Chinese Academy of Sciences, Beijing, China

<sup>5</sup>Department of Plant Biology, College of Biological Sciences, University of California, Davis, CA, USA

Received 26 June 2021;

revised 17 September 2021;

accepted 2 October 2021.

\*Correspondence (Tel: +86 551 657 86401; Fax: +86 551 657 86765; Email: zhliang@ahau.edu.cn)

<sup>†</sup>These authors contributed equally to this work.

**Keywords:** *Camellia sinensis* L., theanine, *CsAlaDC*, ethylamine, biosynthesis, *CsTSI*.

Tea, the second most popular nonalcoholic beverage consumed worldwide, is favoured by billions of consumers due to its special flavour and numerous health benefits (Higdon and Frei, 2003). Theanine, a unique secondary metabolite in the tea plant (*Camellia sinensis* L.), confers the umami taste of the tea infusion. Theanine also has many physiological beneficial effects, including promoting relaxation, improving sleep quality and immunity and protecting the cardiovascular system (Kanarek *et al.*, 2011).

Theanine only accumulates at a high level in tea plants (Tadahiro and Shinsuke, 1984). This might be controlled by the specific presence of ethylamine in tea plants (Cheng *et al.*, 2017), as theanine is primarily biosynthesized from ethylamine and glutamate by theanine synthetase in tea roots, with ethylamine being synthesized from alanine, by alanine decarboxylase (*AlaDC*) (Figure 1a,b), and *CsAlaDC* being specifically expressed in tea roots (Figure 1c). Although *CsAlaDC* exhibited *AlaDC* activity, *in vitro* (Bai *et al.*, 2019), the *in vivo* role in tea plants has not been characterized.

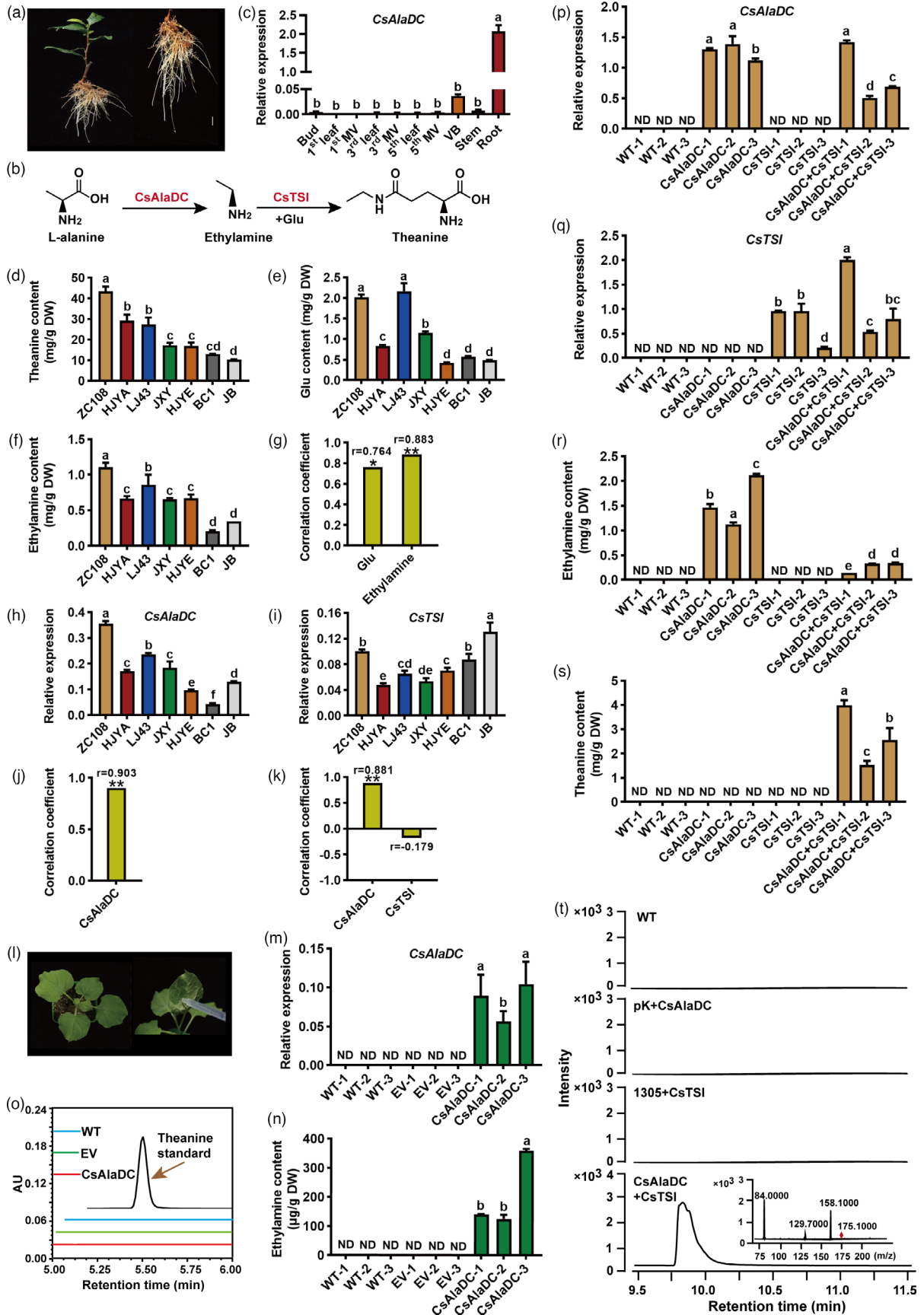
Theanine content varies greatly among the different cultivars, and theanine levels appear to be genetically regulated in tea leaves (Fang *et al.*, 2021). To investigate the *in vivo* role of ethylamine in theanine accumulation, we quantified the key metabolites in the theanine metabolic pathway, in the roots of seven tea plant cultivars (Figure 1d–f). As shown in Figure 1d, theanine contents in the roots of these cultivars varied greatly. Importantly, the ethylamine contents exhibited a similar pattern as the theanine contents (Figure 1f). The correlation coefficient between the contents of ethylamine and theanine was 0.883 ( $P < 0.01$ ) (Figure 1g). Taken together, these findings provided *in vivo*

evidence for the pivotal role of ethylamine in determining theanine accumulation in tea plants.

Next, we examined *CsAlaDC* expression in the roots of the seven cultivars (Figure 1h,i). Here, we found that its expression level was highly and positively correlated with the ethylamine contents, and the correlation coefficient reached 0.903 ( $P < 0.01$ ) (Figure 1j). We also analysed the correlation between *CsAlaDC* expression and theanine content. Our findings indicated that *CsAlaDC* expression, in roots, was highly and positively correlated with the theanine contents, and the correlation coefficient was 0.881 ( $P < 0.01$ ) (Figure 1k). Unexpectedly, the *CsTSI* expression level was not correlated with the theanine contents (Figure 1k). Taken together, these results suggested that *CsAlaDC* expression plays a critical role in determining theanine accumulation in roots of tea plants.

We used the *Nicotiana benthamiana* transient expression system to further characterize the function of *CsAlaDC*, *in planta*. To this end, *A. tumefaciens* strain GV3101 (pSoup-p19), carrying pCAMBIA1305-*CsAlaDC*-*GFP* plasmid, was infiltrated into leaves of 5-week-old *N. benthamiana* plants (Figure 1l); the pCAMBIA1305 empty vector was used as the control (EV). We detected *CsAlaDC* expression at the mRNA level (Figure 1m) by qRT-PCR, and a high level of ethylamine (Figure 1n;  $>100 \mu\text{g/g}$  dry weight) was also identified in the *CsAlaDC*-expressing tobacco leaves. As anticipated, no ethylamine product was detected in WT tobacco leaves and those infiltrated with EV. Taken together, these findings offer support for the hypothesis that *CsAlaDC* has the capacity to synthesize ethylamine, *in planta*.

Theanine is highly demanded, by the market, due to its health effects and medicinal value, as a food constituent, in cosmetics and in other fields (Cheng *et al.*, 2017). The theanine obtained both by direct extraction and chemical synthesis is of very poor quality (Gu *et al.*, 2004). In addition, plant cell culture is disadvantaged by high cost, poor genetic stability and the low content of metabolites, hampering its use for industrial theanine production. An alternative, and intriguing possibility, would be to synthesize theanine in other crops to improve their health-promoting effects of foods. Until now, however, the synthesis of theanine in non-tea plants through synthetic biology has not been achieved. Tobacco (*N. benthamiana*) is generally used as the



**Figure 1** *CsAlaDC* expression was highly correlated with ethylamine and theanine contents in different tea plant cultivars and conferred a high level of theanine accumulation when co-expressed with *CsTST1* in *Nicotiana benthamiana*. (a) Roots harvested for analysis. (b) Theanine biosynthesis pathway in the tea plant. *CsAlaDC*, alanine decarboxylase; *CsTST1*, theanine synthetase. (c) Tissue-specific expression pattern of *CsAlaDC*. MV, major vein; VB, vascular bundle, peeled from the stem. (d–f) Theanine (d), glutamate (e), and ethylamine (f) contents in the roots of 7 tea plant cultivars. ZC108, Zhongcha 108; HJYA, Huangjiyina; LJ43, Longjing 43; JXY, Jingxiangyu; HJYE, Huangjiyine; BC1, Baicha 1; JB, Jibai. (g) The correlation coefficient of glutamate and ethylamine contents with theanine contents. \* $P < 0.05$ , \*\* $P < 0.01$ . (h, i) The relative expression of *CsAlaDC* (H) and *CsTST1* (I). *CsGAPDH* was used as an internal control. (j, k) The correlation coefficient of *CsAlaDC* expression levels with ethylamine contents (j), and the correlation coefficient of *CsAlaDC* and *CsTST1* expression levels with theanine contents (k). \*\* $P < 0.01$ . (l–o) Ectopic expression of *CsAlaDC* in *N. benthamiana* leaves. WT, wild-type *N. benthamiana* without infiltration. EV, empty vector control; *CsAlaDC*, overexpression of *CsAlaDC* in *N. benthamiana*. *NbGAPDH* was used as an internal control. (p–t) Theanine contents in *N. benthamiana* leaves co-expressing *CsAlaDC* and *CsTST1*. *CsAlaDC*, pK7WGF2+pCAMBIA1305-*CsAlaDC*; *CsTST1*, pCAMBIA1305+pK7WGF2-*CsTST1*; *CsAlaDC*+*CsTST1*, pCAMBIA1305-*CsAlaDC*+pK7WGF2-*CsTST1*. Theanine in leaves of *N. benthamiana* was identified by mass chromatogram (UPLC-QQQ-MS). Characteristic theanine ions are located at  $m/z$  158. Data represent means  $\pm$  SD ( $n = 3$ ), significant difference ( $P < 0.05$ ) was labelled with different letters, according to Duncan's multiple range test.

model plant for synthetic biology research (Forestier *et al.*, 2021; Li *et al.*, 2019). Thus, we felt it would be important to synthesize theanine in tobacco, as a model for theanine biosynthesis in non-tea plants.

Given the biosynthesis of ethylamine in tobacco, we speculated whether theanine could be produced in the tobacco leaves in the presence of ethylamine. Unexpectedly, theanine production was not detected (Figure 1o), indicating that the presence of ethylamine did not lead to theanine synthesis, at least not in detectable quantities.

We then speculated that the combination of *CsAlaDC* and *CsTST1*, in tobacco leaves, could synthesize theanine. Based on this notion, we co-infiltrated *A. tumefaciens* strains carrying pCAMBIA1305-*CsAlaDC* and pK7WGF2-*CsTST1* plasmids (*CsAlaDC* + *CsTST1*) into tobacco leaves. *A. tumefaciens* strains containing pCAMBIA1305 and pK7WGF2-*CsTST1* (*CsTST1*) and pK7WGF2 and pCAMBIA1305-*CsAlaDC* (*CsAlaDC*) were infiltrated as the controls. Both *CsAlaDC* and *CsTST1* were being expressed in the infiltrated tobacco leaves (Figure 1p,q). Ethylamine was produced in *CsAlaDC*- and *CsAlaDC*+*CsTST1*-expressing leaves (Figure 1r). High levels of theanine were produced in leaves co-expressing *CsAlaDC* and *CsTST1*, and the theanine content reached to ~4 mg/g (Figure 1s,t), which is comparable with the theanine contents in tea plant leaves. Thus, theanine biosynthesis requires both the presence of ethylamine and the co-action of *CsAlaDC* and *CsTST1*. To our knowledge, this is the first report of theanine biosynthesis, by synthetic biology, in a non-tea plant.

In summary, we established that *CsAlaDC* works coordinately with *CsTST1* in determining the unique high theanine accumulation in the roots of tea plants. More importantly, we reported the first biosynthesis of theanine in a model plant, tobacco. Theanine production in this system does not require the provision of additional substrates, thereby greatly reducing costs and avoids causing environmental pollution. As theanine confers the umami taste, but also has various health effects, engineering its synthesis in non-tea plants, including crops, may well contribute to improve the health-promoting quality of the foods derived from such crops, as well as meeting the market demand for theanine.

## Acknowledgement

This work was supported by grants from the National Natural Science Foundation of China (32072624, 31770731), the Base of

Introducing Talents for Tea Plant Biology and Quality Chemistry (D20026) and Outstanding Youth Project of the Natural Science Foundation of Anhui Province (2008085J18).

## Conflict of interest

The authors declare that they have no conflict of interest.

## Author contributions

ZZ, TY, YW and SB conceived the study and designed the experiments; BZ, JG, CD, FL, SQ and SL carried out the experiments; BZ and ZZ wrote the manuscript, with editing by WJL; SB and WJL provided instructive comments. All authors reviewed and approved the final manuscript.

## References

- Bai, P., Wei, K., Wang, L., Zhang, F., Ruan, L., Li, H., Wu, L. and *et al.* (2019) Identification of a novel gene encoding the specialized alanine decarboxylase in tea (*Camellia sinensis*) plants. *Molecules*, **24**(3), 540.
- Cheng, S., Fu, X., Wang, X., Liao, Y. and Yang, Z. (2017) Studies on the biochemical formation pathway of the amino acid L-theanine in tea (*Camellia sinensis*) and other plants. *J. Agr. Food Chem.*, **65**(33), 7210–7216.
- Fang, K., Xia, Z., Li, H., Jiang, X., Qin, D., Wang, Q., Wang, Q. *et al.* (2021) Genome-wide association analysis identified molecular markers associated with important tea flavor-related metabolites. *Hortic. Res.*, **8**(1), 42.
- Forestier, E., Czechowski, T., Cording, A.C., Gilday, A.D. and Graham, I.A. (2021) Developing a *Nicotiana benthamiana* transgenic platform for high-value diterpene production and candidate gene evaluation. *Plant Biotechnol. J.*, **19**(1), 98–108.
- Gu, H., Jiang, Y. and Wang, J. (2004) A practical synthesis of ethyl-L-glutamine (L-Theanine). *Org. Prep. Proced. Int.*, **36**(2), 182–185.
- Higdon, J.V. and Frei, B. (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.*, **43**(1), 89–143.
- Kanarek, R., Mahoney, C., Brunye, T. and Giles, G. (2011) Theanine mitigates caffeine-accentuated stress response and reduces attentional processing biases following exposure to stress. *Eur. J. Pharmacol.*, **668**, e16.
- Li, J., Mutanda, I., Wang, K., Yang, L., Wang, J. and Wang, Y. (2019) Chloroplast metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in *Nicotiana benthamiana*. *Nat. Commun.*, **10**(1), 4850.
- Tadahiro, N. and Shinsuke, S. (1984) Differences in caffeine, flavanols and amino acids contents in leaves of cultivated species of *Camellia*. *Japan J. Breed.*, **34**(4), 459–467.