Brief Communication

CsMYB184 regulates caffeine biosynthesis in tea plants

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As the most well-known and globally consumed central nervous system stimulant, caffeine is a purine alkaloid natural product usually derived from tea and coffee. Caffeine has a wide range of health benefits on the human body, and plays crucial roles in pollination, resistance to herbivore attacks, and pathogen infections in plants (Zhao et al., 2020). While caffeine biosynthetic pathways have been extensively studied in tea (Camellia sinensis) and coffee plants, the regulation of caffeine biosynthesis is not understood (Zhao et al., 2020).

Tea Caffeine Synthase1 (TCS1) is the first N-methyltransferase gene reported in the tea plant, possessing the 1-N methyltransferase activity responsible for converting theobromine to caffeine (Kato et al., 2000). Studies on the structure-activity of TCS1 and genetic variations in the TCS1 gene of tea plant populations have supported that TCS1 is a determination enzyme for caffeine content (Jin et al., 2016). To explore the regulation of caffeine biosynthesis, 23 candidate transcription factors (TFs) from Weighted Gene Co-expression Network Analysis were screened in a luciferase reporter gene activation system driven by the TCS1 promoter (Figure S1). MYB184 (TEA029017) showed the highest TCS1 promoter activation with 4.7-fold (Figure 1a). Yeast one-hybrid assay showed that the −828 to −1670 bp region of TCS1 promoter, which contains an MYBCORE and a fused MYB1AT-MYBPLANT, was critically required for MYB184 recognition (Figure 1b). In planta promoter, trans-activation assays further confirmed the regions required for binding to and activating of the TCS1 promoter by MYB184 to be between −1596 and −1670 bp (Figure 1c). EMSA assay was performed to further validate the binding of MYB184 to the fused MYB1AT-MYBPLANT motif in vitro (Figure 1d-f).

We then examined the function of MYB184 in regulating caffeine synthesis in tea plants. An antisense oligodeoxynucleotide (asODN) interference experiment was performed with tea plant shoot tips to knock down MYB184 expression (MYB184-KD) (Figure 1g). Accordingly, caffeine contents and the expression of TCS1 were significantly reduced in MYB184-KD shoot tips compared with the senseODN control (Figure 1h-j). However, overexpression of MYB184 (MYB184OE) in tea plant transgenic hairy root lines significantly up-regulated TCS1 transcription and thereby increased the caffeine contents as compared with wild-type root controls (Figure 1j-m).

KeKecha (Camellia ptilophylla, KKC in short), belonging to the Thea section, had significantly lower caffeine but higher theobromine (Figure 1n). Although a previous study showed that TCS1 in KKC had lower NMT activity compared with TCS1 in modern tea cultivars (Jin et al., 2016), we detected a significantly lower expression level of TCS1 in KKC than in other tea cultivars (Figure 1o). To understand why TCS1 in KKC is down-regulated, we cloned and compared the promoter sequences of TCS1 from KKC and from SCZ. However, the alignment of the promoter sequences did not show critical Indels or SNPs on MYB binding sites (Figure 1p). On the other hand, transcriptome analyses showed that only MYB184 expression level was significantly lower by ~14-fold in KKC than in other tea cultivars (Figure 1q,r). We thus proposed that the lower MYB184 transcript level in KKC might be the cause of the reduced TCS1 expression level.

We further cloned the promoter of the MYB184 gene from KKC (proMYB184KKC), and compared it with those from other tea cultivars. A 437-bp long terminal repeat (LTR) insertion was identified only in the proMYB184KKC at the site of ~982 bp, but not in these promoters from other tea cultivars, as verified with both MYB184 promoter cloning and detection with PCR primers specific for the LTR insertion (Figure 1s). In tea plants, LTR insertion in a gene usually leads to suppression of the gene expression (Xia et al., 2020), which may explain the lower expression level of MYB184 in KKC than in other tea cultivars. Indeed, GUS reporter assays showed that the proMYB184KKC with the LTR insertion exhibited clearly lower promoter activity than four representative promoters without the LTR insertion from tea cultivars (Figure 1t). We thus concluded that the LTR insertion in the promoter of MYB184 resulted in suppressed MYB184 expression, leading to the lower TCS1 transcript level and thereby lower caffeine contents in KKC.

To expand MYB184 activation of TCS1 to other Camellia species, we examined several other wild tea relatives that are known to contain significantly lower levels of caffeine compared with modern tea cultivars (Figure 1u). They also have lower TCS1 and MYB184 expression levels compared with modern tea cultivars containing higher levels of caffeine (Figures 1v,w and S2). This further supports the indispensable role of MYB184 in activation of TCS1 gene expression and caffeine biosynthesis in C. sinensis.

In summary, we characterized MYB184 as the major activator of TCS1 and caffeine production in tea plants. An LTR insertion in the MYB184 promoter in wild tea C. ptilophylla explained its low TCS1 expression level and caffeine content. Our study may offer a
molecular tool for breeding low-caffeine tea varieties to meet the market demands.

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Conflicts of interest

The authors declare no conflict of interest.
Figure 1 CsMYB184 positively regulates caffeine biosynthesis in tea plants. (a) Screening for trans-activation of Tea caffeine synthase (TCS1) promoter with 23 TEs in luciferase reporter assay in Arabidopsis protoplasts with Renilla luciferase as a reference. (b) MYB184 binds to the TCS1 promoter. Based on the distribution of cis-elements, the full-length TCS1 promoter (P1, −1 to −1670 bp) and three truncated versions, named P2 (−1 to −1596 bp), P3 (−1 to −828 bp), and P4 (−1 to −176 bp) were cloned in front of the HIS gene respectively. (c) Promoter activation of the TCS1-P1, -P2, -P3, and -P4 promoters by MYB184. (d) The putative MYB binding sites in TCS1 promoter for EMSA assay. (e) Recombinant GST-MYB184 fusion protein purified from Escherichia coli BL21 cells. The arrow indicates the target protein. (f) EMSA assay of MYB184 binding to TCS1 promoters. Competitor represents the putative motif without the biotin label. The concentration of competitors with different rations with a biotin-labelled motif is 5×, 10×, or 50×. The arrow indicates the target biotin signal. (g) Down-regulation of MYB184 in tea plant shoot tips treated with asODN-MYB184. (h) Repressed caffeine biosynthesis in MYB184-KD tea shoot tips compared with sODN. (i) Effect of MYB184-KD on TCS1 expression in tea plant shoot tips. (j) Overexpression of MYB184 in transgenic tea hairy roots (n = 4). (k) HPLC traces of caffeine from tea plant hairy roots overexpressing MYB184 (MYB184OE) and GFP (control). (l, m) Ectopic expression of MYB184 promoted caffeine biosynthesis (l) and TCS1 expression level (m) in MYB184OE hairy roots compared with GFP hairy roots. (n) HPLC analysis of purine alkaloids in young leaves of Shuchazao and C. ptilophylla (KKC). (o) Comparison of TCS1 expression levels in young leaves of KKC and other ten cultivars. SCZ, Shuchazao; CBL, Chunbolv; FZ2H, Fuzaoerhao; ZC102, Zhongcha102; BHZ, Baihaozao; ZY, Ziyan; ZN117, Zhenong117; RX, Ruixiang, BS, Benshan; XH, Xiuong. (p) Distribution of MYB binding site remarked using a red box on the cultivars. SCZ, Shuchazao; CBL, Chunbolv; FZ2H, Fuzaoerhao; ZC102, Zhongcha102; BHZ, Baihaozao; ZY, Ziyan; ZN117, Zhenong117; RX, Ruixiang, BS, Benshan; XH, Xiuong. (q) Down-regulation of MYB184 expression in tea plant shoot tips treated with asODN-MYB184. (r) Fold-change of MYB184 expression level in KKC and some tea cultivars. The triangle indicates LTR insertion; orange rectangles indicate exons. The forward (F) and reverse (R) primers used to detect the LTR insertion were also marked. (t) Different MYB184 promoter activities from KKC and tea cultivars (SCZ, ZC102, BHZ, BS) were verified in tobacco leaves. (u) Caffeine contents in tea cultivars (n = 50) and wild tea relatives in Thea section (including Camellia taliensis, Camellia gymnogyna, Camellia crassicolumna, and Camellia ptilophylla). (v, w) Expression patterns of MYB184 (v) and TCS1 (w) in tea cultivars and wild tea relatives. Statistical significance for each comparison is indicated (t-test: *P < 0.05, **P < 0.01). CsACTIN and CsGAPDH were introduced to normalize the expression.

Author contributions
J.Z. conceived the research. P.H.L., J.M.F., Z.L.Y., J.Y.X., Y.H.S., Y.R.Z., D.K.T., P.L., H.Z, and W.T. performed experiments and P.H.L., J.Z., S.C.W., and F.R.A. wrote the manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Caffeine regulator screening in tea plants.
Figure S2 Specific caffeine biosynthesis pathway and analyses of MYB184 promoters in tea plants.