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Differential gene expression in tea (*Camellia sinensis* L.) calli with different morphologies and catechin contents

Dongqing Yang[a,1], Yajun Liu[a,1], Meilian Sun[a], Lei Zhao[a], Yunsheng Wang[b], Xiaotian Chen[b], Chaoling Wei[a], Liping Gao[b,*], Tao Xia[a,**]

[a] *Key Laboratory of Tea Biochemistry & Biotechnology, Ministry of Education in China, Anhui Agricultural University, 130 West Changjiang Rd., Hefei, Anhui 230036, China*
[b] *School of Life Science, Anhui Agricultural University, 130 West Changjiang Rd., Hefei, Anhui 230036, China*

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ABSTRACT

Tea (*Camellia sinensis*) is a commercially important crop that contains valuable secondary metabolites. To understand the molecular regulation of secondary metabolism in tea, we selected and analyzed two cell lines of tea callus (Yunjing63Y and Yunjing63X) that showed different morphological characteristics and catechin contents. Yunjing63Y callus was yellow and tight, while yunjing63X callus was white and loose. HPLC analyses showed that Yunjing63Y contained 3.71 times higher levels of catechins than Yunjing63X. Using cDNA amplified fragment-length polymorphism (cDNA-AFLP) we identified 68 genes that were differentially expressed between the two lines. Of the 68 differentially expressed ESTs, 40 showed higher expressions in Yunjing63Y and 28 showed higher expressions in Yunjing63X. BLASTX comparisons classified these ESTs into seven functional groups; phenylpropanoid metabolism (2.9%), UDPG-dependent glucosyl transferase (8.8%), transcription factors (11.8%), transporters (13.2%), signal transduction (19.1%), other metabolism (26.5%), and unknown (17.7%). We used qRT-PCR to validate the expression of genes and ESTs, and found that genes associated with flavan-3-ols biosynthesis and metabolism were expressed at higher levels in Yunjing63Y than in Yunjing63X. In addition, the expression of ESTs associated with flavonoid biosynthesis, regulation and transport were higher in Yunjing63Y than in Yunjing63X. The full-length cDNA of a EST coding for a putative MYB transcription factor was amplified using rapid amplification of cDNA ends (RACE). The resulting 1270 bp long cDNA, named CsMYB1, contained a 933-bp ORF encoding a 310-amino acid protein with a predicted molecular weight of 105.27 kDa and a predicted isoelectric point of 4.85 and showed highest homology to plant MYBs likely involved in stress signaling.

Introduction

Camellia sinensis has been used for tea beverage since 3000 B.C. (Mondal et al., 2004), which consists of the leaf and bud of the plant Camellia *sinensis,* is the oldest non-alcoholic beverage in the world, having been consumed socially and habitually since 3000 B.C. (Mondal et al., 2004). Recent epidemiological studies suggest that green tea has diverse biological activities, including antioxidant, antimutagenic, anticarcinogenic, antibacterial, antiviral, and anti-fibrotic activities. In addition, it has been shown to alleviate the symptoms of hypertension, reduce the risk of cardiovascular disease, improve oral health, protect against solar ultraviolet radiation, control body weight, improve glucose tolerance and insulin sensitivity, increase bone mineral density, and maintain neuroprotective activity (Cabrera et al., 2006). Besides green tea, 'semi-fermented' oolong tea and 'fermented' black tea are also beneficial to human health. In tea, there are three major components that affect human health; xanthic bases (caffeine and theophylline), essential oils, and polyphenolic compounds (Cabrera et al., 2006). Among these components, polyphenolic compounds have received a great deal of

Abbreviations: 4CL, 4-Coumaroyl:CoA ligase; ANR, Anthocyanidin reductase; ANS, Anthocyanidin synthase; C, (+)Catechin; C4H, Cinnamate 4-hydroxylase; CHI, Chalcone isomerase; CHS, Chalcone synthase; COMT, Caffeic acid O-methyltransferase; DFR, Dihydroflavonol 4-reductase; EC, (−)-Epicatechin; ECG, (−)-Epicatechin-3-gallate; EGC, (−)-Epigallocatechin; EGCG, (−)-Epigallocatechin-3-gallate; F3′5′H, Flavonoid 3′,5′hydroxylase; F3′H, Flavonoid 3′hydroxylase; F3H, Flavanone 3-hydroxylase; GC, (+)Gallocatechin; GST, Glutathione-S-transferase; LAR, Leucoanthocyanidin reductase; PAL, Phenylalanine ammonia lyase; UFGT, UDP-glucose: flavonoid 3′-O-glucosyltransferase; PAs, proanthocyanidins.

* Corresponding author. Tel.: +86 551 5786232; fax: +86 551 5785729.
** Corresponding author. Tel.: +86 551 5786003; fax: +86 551 5785729.
E-mail addresses: qingqingy2110@yahoo.com.cn (D. Yang), liuyajun1228@163.com (Y. Liu), meiliansun@163.com (M. Sun), ganxiezhenshi@163.com (L. Zhao), wang_li09213@sina.com (Y. Wang), cxt86@163.com (X. Chen), weichl@ahau.edu.cn (C. Wei), gaolp62@126.com (L. Gao), xiatao62@126.com (T. Xia).
[1] These authors contributed equally to this work.

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attention because they significantly affect human health and have diverse physiological functions in the plant.

Flavan-3-ols (catechins), which are abundant in young leaves and shoots of the tea plant, account for approximately 70–80% of tea polyphenols. The biosynthesis of flavonoids in plants has been investigated intensively at the biochemical and genetic levels over recent decades (Winkel-Shirley, 2001; Dixon et al., 2005). Recently, there have been considerable advances in research on catechin biosynthesis in tea plants. Some of the genes in the tea catechin biosynthesis have been cloned, and the relationship between expression of these genes and accumulation of catechins has been investigated (Punyasiri et al., 2004; Singh et al., 2008, 2009a,b; Eungwanichayapant and Popluechai, 2009; Ashihara et al., 2010). However, the mechanisms involved in biosynthesis of catechins, especially the gene regulatory network, are still poorly understood.

To understand the gene regulatory network of catechin biosynthesis in tea, it would be helpful to study differentially expressed genes in different experimental materials, such as genetic mutants and leaves at different developmental stages. Suspension culture systems provide a more controllable model for studying catechin biosynthesis. Many studies have been carried on the production of tea catechins using such cell cultures (Koretskaya and Zaprometov, 1975; Zaprometov et al., 1986; Zagoskina et al., 1990; Shibasaki-Kitakawa et al., 2003).

In this study, we selected two lines of callus, Yunjing63X and Yunjing63Y, which showed different morphological characteristics and catechin contents. Using these two lines as experimental materials, we examined differential gene expression using cDNA amplified fragment-length polymorphism (cDNA-AFLP) and quantitative real-time polymerase chain reaction (qRT-PCR) techniques. In addition, we isolated a putative v-myb avian myeloblastosis viral oncogene homolog (MYB) transcription factor gene, *CsMYB*1.

Materials and methods

Materials and culture conditions

Tea plants [*Camellia sinensis* (L.) O. Kuntze] grown in the botanical garden of Anhui Agricultural University, Hefei, P. R. China were used to get shoot tip explants. The explants were sterilized and cultured at $25 \pm 2\,^{\circ}C$ in the dark in B_5 culture medium (pH 5.5) containing 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} kinetin (KT), 30 mg L^{-1} sucrose, and 0.7% (w/v) agar. Calli appeared a few weeks later and were subcultured under the same conditions until they became loose. Yellowish, loose callus was selected and subcultured every 20 days by transferring approximately 5 g calli to 100 mL of the liquid medium, containing the same components except for agar, in 250 mL Erlenmeyer flasks. This callus was stable during long-term subculturing, and was designated as Yunjing63. The calli were selected repeatedly according to their morphology and color. The yellow and substantial callus was designated as Yunjing63Y, while the white and loose callus was designated as Yunjing63X.

Polyphenol extraction and quantitative analysis

Catechins and flavonols were extracted from calli and analyzed according to the methods described by Liu et al. (2009). To extract catechins and flavonols, 3 g of calli or leaves was crushed in liquid nitrogen and macerated with 10 mL 95% ethanol. The ethanol extract was centrifuged at 4000 × *g* for 15 min to separate the supernatant and precipitate. The supernatant was evaporated and then dissolved in hot water, and then the water solution was extracted three times with ethyl acetate. The organic phase was evaporated and dissolved in 1 mL methanol. The catechins and flavonols in methanol extracts were analyzed by HPLC.

All extracts were filtered through a 0.22 μm membrane before injected into a Phenomenex Synergi 4 μm Fusion-RP80 column (250 mm × 4.6 mm) with detection at 280 nm for catechins and 340 nm for flavonols using an HPLC-UV detector (Waters 2478, Waters Instruments). The binary solvent system consisted of 1% (v/v) acetic acid (A) and 100% acetonitrile (B). After injection of the sample (5 μL), an isocratic flow was initiated at a flow rate of 1.0 mL/min for 10 min, and then the following linear gradient was applied: solvent B from 0 to 21% over 40 min, then from 21 to 40% between 50 and 55 min, and from 40 to 0% between 56 and 58 min. Peaks were identified by comparison of retention times to those of authentic catechin standards. The concentrations of catechins and flavonols in extracts determined by HPLC were calculated using an external standard method and area of the peak, respectively.

Analysis of PAs followed the methods of Pang et al. (2008) with some modifications. Fresh leaves and calli (0.5 g each) were crushed in liquid nitrogen and extracted with 3 ml methanol by sonication at room temperature for 10 min, followed by centrifugation at 4000 × *g* for 15 min, and the residues were re-extracted twice as above. The pooled supernatants were extracted three times with chloroform. The supernatants were pooled in a total volume of 10 mL.

Total PA amount was the sum of soluble and insoluble PA detected from the plant sample. For soluble PA analysis, 1 mL water was added to 1 mL supernatants extract, followed by addition of 2 mL chloroform to remove chlorophyll, 0.5 mL aqueous phase was added to 3 ml *n*-butanol–HCl (95/5, v/v), then incubated at 95 °C for 1 h. The supernatant was cooled to room temperature, and the absorbance at 550 and 600 nm were recorded. The control treatment was performed under the same conditions without boiling. Relative anthocyanin concentrations were calculated as absorbance at 550 nm minus absorbance at 600 nm. Absorbance values were converted into PA equivalents using a standard curve of cyanidin chloride (CYA, AXXORA, Lausen, Switzerland). For insoluble PA analysis, the residues were added to 3 mL *n*-butanol-HCl (95/5, v/v), then incubated at 95 °C for 1 h. The detection method used for soluble PAs in the supernatant was applied as described above.

For anthocyanins analysis, extraction of anthocyanins in leaves and calli followed the method of Pang et al. (2008). The absorbance values of the extract were recorded at 530 nm and 600 nm. Relative anthocyanin concentrations were calculated as absorbance at 530 nm minus absorbance at 600 nm. Total anthocyanin content was calculated based on a cyanidin-3-O-glucoside standard (Xin-Ran Biological, Shanghai, China). Lignin content was determined with a modified Klason, where extracted ground leaf tissues (0.4 g) or calli (0.4 g) were treated with 6 ml of 72% H_2SO_4 according to Coleman et al. (2006).

The above result values are means of three replicates. Results represent mean ± SD ($n = 3$).

Total RNA isolation and cDNA-AFLP analysis

Total RNA was isolated from calli using the cetyl-trimethylammonium bromide-LiCl (CTAB-LiCl) method (Jaakola et al., 2001), and assessed by UV spectrophotometry and agarose gel electrophoresis. The purity of RNA was determined by absorbance ratios (A260/A280 and A260/A230) and RNA integrity was evaluated by comparing the intensity of the 28S/18S rRNA ratio.

The cDNA-AFLP analyses were performed according to Bachem et al. (1996) and van Raemdonck et al. (2005), with minor modifications. First and second cDNA strands were synthesized with an M-MLV RTase cDNA Synthesis Kit (Takara, Japan). Double-stranded cDNA was digested with the restriction enzymes *Mse*