Expression for Caffeine Biosynthesis and Related Enzymes in *Camellia sinensis*

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Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that is present in high concentrations in the tea plant *Camellia sinensis*. Caffeine synthase (CS, EC 2.1.1.160) catalyzes the *S*-adenosyl-L-methionine-dependent *N*-3- and *N*-1-methylation of the purine base to form caffeine, the last step in the purine alkaloid biosynthetic pathway. We studied the expression profile of the tea caffeine synthase (TCS) gene in developing leaves and flowers by means of northern blot analysis, and compared it with those of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), chalcone synthase (CHS, EC 2.3.1.74), and *S*-adenosyl-L-methionine synthase (SAMS, EC 2.5.1.6). The amount of TCS transcripts was highest in young leaves and declined markedly during leaf development, whereas it remained constant throughout the development of the flower. Environmental stresses other than heavy metal stress and plant hormone treatments had no effect on the expression of TCS genes, unlike the other three genes. Drought stress suppressed TCS gene expression in leaves, and the expression pattern mirrored that of the dehydrin gene. The amounts of TCS transcripts increased slightly on supply of a nitrogen source. We discuss the regulation of TCS gene expression.

Key words: Caffeine Synthase Gene Expression, Environmental Stress, Leaf and Flower Development, Dehydrin Transcripts

Introduction

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that is present in high concentrations in tea and coffee (Ashihara and Crozier, 1999). The main caffeine biosynthetic pathway is xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine (3,7-dimethylxanthine) → caffeine (Fig. 1) (Suzuki, 1972; Ashihara and Kubota, 1987; Ashihara *et al*., 1996, 1997; Kato *et al*., 1996). This main route in tea and coffee is supported by the substrate specificities of native and recombinant *N*-methyltransferases (NMTs) (Kato *et al*., 1999, 2000; Mizuno *et al.*, 2003a, b; Uefuji *et al.*, 2003), as well as by experiments with radiolabeled precursors. The biosynthesis of caffeine involves three methylation steps. *S*-Adenosyl-L-methionine (SAM), a methyl donor in numerous biological reactions, is the purine-base methyl donor in the biosynthesis of caffeine (Suzuki, 1972). SAM-dependent NMTs play an important role in the regulation of caffeine biosynthesis (Fujimori *et al.*, 1991). The NMTs that are involved in caffeine biosynthesis can be divided into three types according to their substrate specificity. These three types of enzyme and the corresponding genes have already been identified in coffee. The first methylation step in the biosynthetic pathway of caffeine, from xanthosine to 7-methylxanthosine, is catalyzed by 7-methylxanthosine synthase (Mizuno *et al.*, 2003b; Uefuji *et al.*, 2003). Recent studies on the chemical structure of the protein have shown that this enzyme has the dual functions of methyl transfer and nucleoside cleavage (McCarthy and McCarthy, 2007). The 7-methylxanthosine synthase gene(s) has (have) not yet been cloned in tea (*Camellia sinensis*). The last two steps of caffeine biosynthe-

Abbreviations: ABA, abscisic acid; cDNA, complementary DNA; CHS, chalcone synthase; CS, caffeine synthase; MeJA, methyl jasmonate; NMT, *N*-methyltransferase; PAL, phenylalanine ammonia lyase; PCR, polymerase chain reaction; SA, salicylic acid; SAM, *S*-adenosyl-L-methionine; SAMS, *S*-adenosyl-L-methionine synthetase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TCS, tea caffeine synthase.

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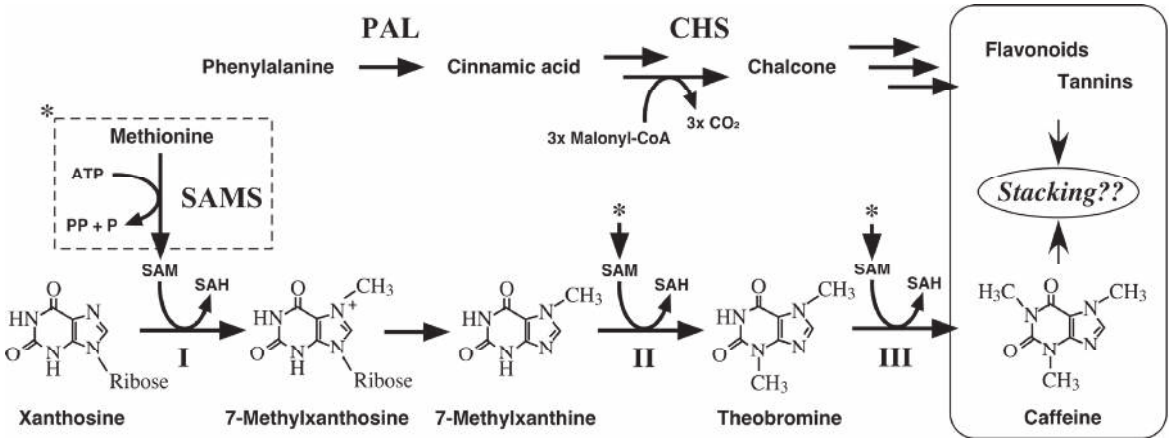

Fig. 1. Pathways for the biosynthesis of caffeine and phenylpropanoids. It is known that phenylpropanoid-containing catechins form complexes with caffeine (Hayashi *et al.*, 2004).

sis are catalyzed by caffeine synthase (CS) (Kato *et al.*, 1999, 2000; Mizuno *et al.*, 2003a; Uefuji *et al.*, 2003). The first NMT in the caffeine biosynthesis pathway to be biochemically characterized was cloned from young leaves of tea (Kato *et al.*, 1999, 2000). Caffeine synthase catalyzes N-1- and N-3-methylation reactions of mono- or dimethylxanthines. Theobromine synthase, on the other hand, is specific for the conversion of 7-methylxanthine into theobromine (Mizuno *et al.*, 2001; Ogawa *et al.*, 2001; Uefuji *et al.*, 2003). The principal role of theobromine synthase in caffeine-accumulating species is not well understood; however, theobromine synthase is believed to play a key role in theobromine-accumulating species (Yoneyama *et al.*, 2006). Furthermore, the occurrence of transcripts of theobromine synthase genes in purine alkaloid-free *Camellia* plants was demonstrated (Ishida *et al.*, 2009).

High levels of transcripts of tea caffeine synthase (TCS) were detected in young leaves, and much lower amounts were present in old leaves (Kato, 2001). These expression patterns mirror the distribution of caffeine synthase activity in the leaves of tea (Fujimori *et al.*, 1991). On the other hand, the gene expression sequence of developing leaves was not monitored in the earlier study. Moreover, little information is available on the underlying regulation of the expression of the TCS gene. In the present study, we characterized the gene expression pattern of the TCS gene in developing leaves and flowers of tea. Expression of the genes for phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), which are involved in the biosynthesis of phenylpropanoid-containing catechin, and for *S*-adenosyl-L-methionine synthetase (SAMS), which supplies the substrate for NMTs, were also analyzed in developing tissue. Furthermore, we searched for environmental and chemical factors that influence the expression of the TCS gene.

Material and Methods

Plant material

At the Tokyo Metropolitan Agricultural Experimental Station (Tachikawa, Japan) and the Green Tea and Local Products Laboratory of the Saitama Agriculture and Forestry Research Center (Iruma, Japan) the flush shoots of *Camellia sinensis* cv. Yabukita were collected in May through July. The leaves for analysis of the developing stage were collected on May 26, 2004, at the Tokyo Metropolitan Agricultural Experimental Station, and the flower buds for analysis of the developing stage were collected on November 11, 2008, at the Green Tea and Local Products Laboratory. After harvesting, the leaves were frozen in dry ice and stored at −80 °C until required for RNA extraction.

The flush shoots (20–30 cm) were cut off and stood up in a glass beaker containing water or an aqueous solution of the relevant compound. For cold treatment, the plants were transferred to a

cold room equipped with timer-controlled fluorescent lighting. For light and dark treatments, the plants were placed under continuous irradiation (2.6 W m^{-2}) or continuous darkness, respectively. For wounding treatment, two lateral incisions were made on each side of the mid-vein of the leaves with a sterile razor blade. For salt-stress treatment, the plants were exposed to a 400-mM solution of NaCl. For heavy-metal stress-treatment, the plants were exposed to 50 μM $CdCl_2$ solution. For dehydration treatment, the plants were stood up in empty beakers. For rehydration treatment, dehydrated plants were transferred to a beaker containing water. For methyl jasmonate (MeJA) treatment, the plants were sprayed with MeJA dissolved in 0.1% ethanol solution to a final concentration of 50 μM. For salicylic acid (SA) treatment, an 100-μM solution of SA was sprayed onto the plants. For abscisic acid (ABA) treatment, plants were sprayed with ABA dissolved in 1% ethanol solution to a final concentration of 1 mM or 100 μM. For supply of a nitrogen source, the plants were exposed to 1 mM aqueous solutions of $NaNO_3$, NH_4Cl, glutamic acid, or methionine. Upon harvest after the treatments, the leaves were frozen in liquid N_2 and stored at −80 °C until required for RNA extraction.

Cloning of the dehydrin cDNA fragment

Total cellular RNA was extracted from leaves of tea with cetyl(trimethyl)ammonium bromide (CTAB) solution according to the method of Mizuno *et al.* (2003a). First-strand cDNAs were synthesized by using a 3'-rapid amplification of cDNA ends (3'-RACE) core set and oligo-dT three-sites adaptor primer (oligo-dT 3SAP) (Takara Bio Inc., Otsu, Japan) as a primer. To obtain the 3'-regions of dehydrin cDNA, we used Deh1-F (5'-GABAAGATHAARGAGAA-RCT-3') as a primer in 3'-RACE. The polymerase chain reaction (PCR) was conducted in a thermal cycler (PTC-200, Bio-Rad, Hercules, CA, USA) for 30 cycles (60 s at 94 °C, 60 s at 45 °C, and 90 s at 72 °C) with the first-strand cDNAs and primers. The amplified DNA fragment was purified by polyacrylamide gel electrophoresis and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Nucleotide sequencing was carried out using an ABI PRISM 3100 genetic analyzer at the Life Research Support Center of Akita Prefectural University, Japan. Nucleotide and protein sequences were analyzed by computer using GENETYX software (Software Development Co., Tokyo, Japan).

RNA gel blots

Total cellular RNA was extracted from leaves of tea as described above. 5 μg of total RNA from each sample were electrophoresed on 1.2% agarose gels and then blotted onto the membrane as described previously (McMaster and Carmichael, 1977). The RNA was transferred overnight to a Hybond N^+ membrane (GE Healthcare UK Ltd., Buckinghamshire, UK) and crosslinked by UV irradiation. The blots were probed using DNA fragments labeled with [α-^{32}P]deoxycytidine triphosphate ([α-^{32}P]dCTP; 222 TBq $mmol^{-1}$; Institute of Isotopes Co., Ltd., Budapest, Hungary), using a *Bca*BEST™ Labeling kit (Takara Bio Inc.). To detect *TCS* transcripts, a 530-bp *Sty* I fragment containing the central part of the *TCS1*(AB031280) coding region was used as a probe (Kato *et al.*, 2000). cDNA fragments encoding CHS(D26593), PAL(AY694188), and SAMS(AB041534) were obtained by RT-PCR. PCR was performed using the following oligonucleotide primers: 5'-tacatggcaccttcacttg-3' and 5'-gacttcttcctcatctcatc-3' for CHS; 5'-gattgggttatggagagtatga-3' and 5'-ggtgcttcaatttgtgtgtc-3' for PAL; 5'-tccgaatctgtgaatgagggtc-3' and 5'-caaggacatggctgaggggc-3' for SAMS. The amplified DNA fragments were subcloned into pT7blue® vector (Novagen, Madison, WI, USA) and subsequently sequenced for confirmation of sequence integrity. CHS transcripts were detected using an 800-bp *Eco*RI/*Pst* I fragment as a probe. PAL transcripts were detected using a 640-bp *Eco*RI fragment as a probe. SAMS transcripts were detected using a 470-bp *Eco*RI/*Pst* I fragment as a probe. To detect dehydrin transcripts, an *Eco*RI fragment was used as a probe, as described above. The hybridization and washing of membranes was performed as described by Umezawa *et al.* (2002). Positive bands were detected after autoradiography using a BioMax-MS X-ray film (Eastman Kodak, Rochester, NY, USA); the signal was quantified using FLA-2000 (Fuji Photo Film, Tokyo, Japan). Total RNA samples were subjected to electrophoresis in agarose gels and stained with ethidium bromide to compare the rRNA intensities to check for equal RNA loading.

Results

Gene expression of the enzymes involved in the secondary metabolism during leaf and flower development

As shown in Fig. 2, the expression of TCS, CHS, PAL, and SAMS in developing leaves was investigated. The young leaves were divided into four stages on the basis of their weight. Stage 1 consisted of the most recently emerged developing leaves, whereas stage 6 consisted of old leaves that had emerged the previous year. Leaf weights in stage 4 and stage 5 were almost equal, and so we regarded stage 5 as the mature leaves. The amount of TCS transcripts was highest in stage 1 and declined to a half in stage 2. Subsequently, the level of transcripts decreased gradually during stage 2 through stage 5, and remained at 10% of the maximum value in the mature and old leaves. Levels of CHS, PAL, and SAMS transcripts were highest in stage 1, as was the case with TCS; however, no marked decrease between stage 1 and stage 2 was observed. The levels of these three transcripts decreased gradually in the young leaves (stages 1–4) and then decreased markedly in the mature leaves. The variations in mature and old leaves were remarkable in these three transcripts.

The expression of TCS, CHS, PAL, and SAMS were also studied in developing flowers (Fig. 3). Flower buds were divided into four stages on the basis of their fresh weight. Stage 4 was when the buds were in full bloom. The amount of TCS transcripts remained constant throughout development. On the other hand, levels of CHS transcripts were highest in stage 1 and then decreased markedly. PAL showed a strong induction of gene expression during flower development. The expression levels of SAMS transcripts were high in young flower buds and then gradually decreased.

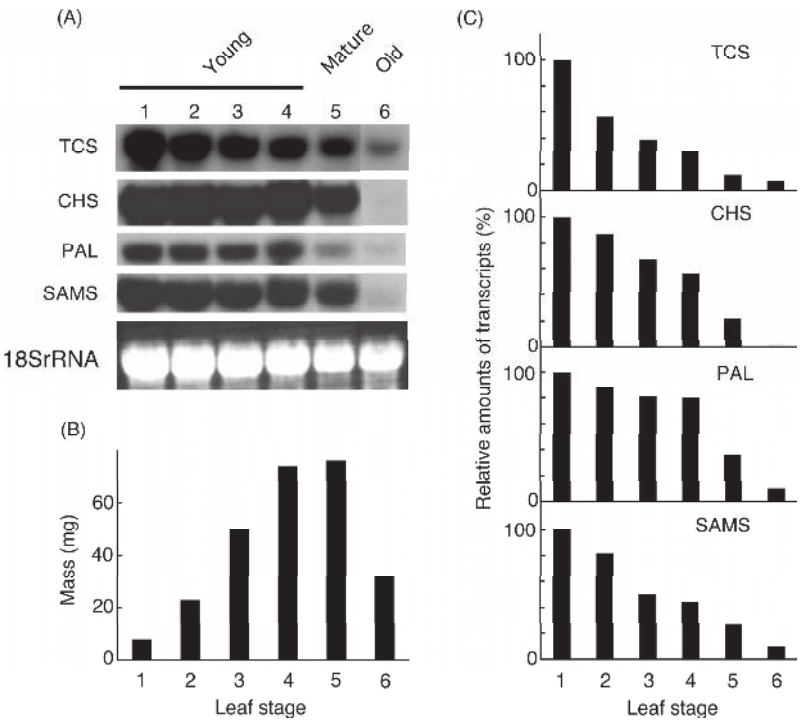

Fig. 2. Northern blot analysis of the expression of TCS, CHS, PAL, and SAMS for various stages of foliar development. Total RNA (5 μg per sample) from developing leaves was subjected to electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA fragments. (A) The expression pattern of TCS, CHS, PAL, and SAMS; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA. (B) Flower mass for various stages of development. (C) The relative amount of hybridized signal in northern blot analysis with FLA-2000.

Gene expression of the enzymes involved in the secondary metabolism response to various environmental stresses and plant hormone treatments

The level of TCS transcripts in leaves from detached plants was similar to the level in leaves from attached plants (data not shown). Therefore the detached plants were subjected to various environmental stress and plant hormone treatments. No treatment significantly altered the expression of TCS transcripts other than the heavy metal stress treatment (Fig. 4). Levels of TCS transcripts became constant 2 h after the initiation of $CdCl_2$ supply, and disappeared 24 h after the treatment. Levels of CHS, PAL, and SAMS transcripts also decreased 24 h after the initiation of $CdCl_2$ supply. The expression patterns of the four genes during exposure to $CdCl_2$ were quite similar, suggesting that this suppression was not a specific blocking of the transcription of one or more genes.

Continuous illumination induced the accumulation of CHS and PAL transcripts to a level five times higher than the control 24 h after initiation of the treatment. A low temperature also induced the accumulation of CHS and PAL transcripts. Two hours after wounding, PAL and SAMS transcripts were detected at levels twice those of the control. SA induced a transient increase in the level of

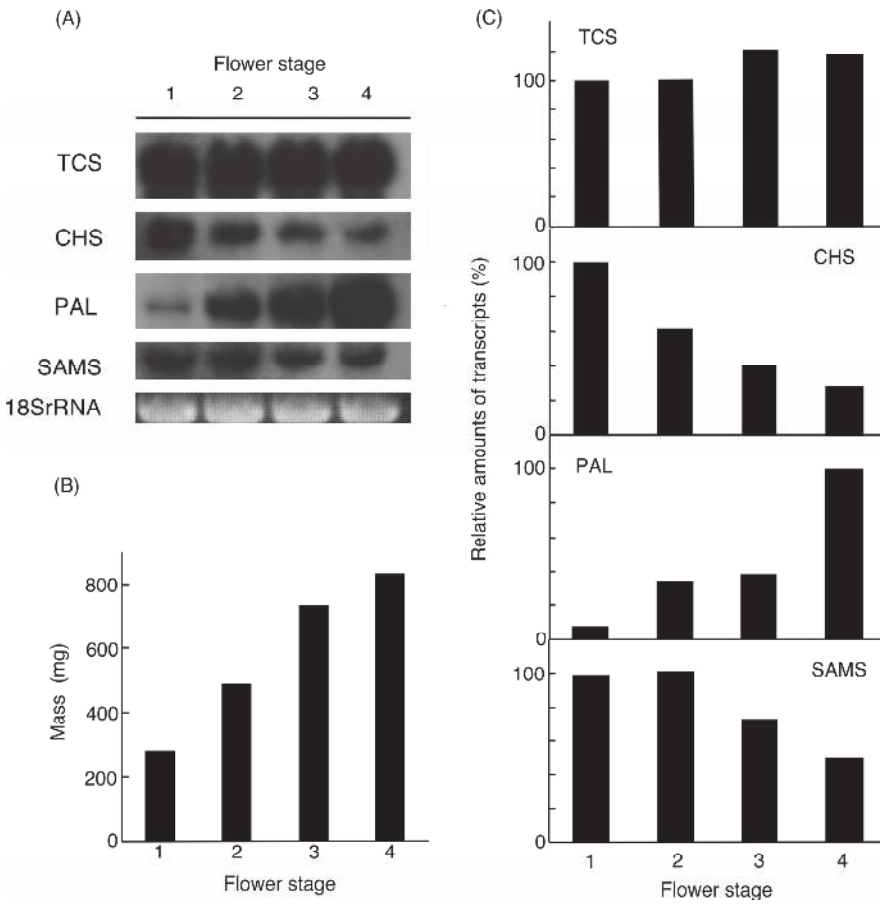

Fig. 3. Northern blot analysis of the expression of TCS, CHS, PAL, and SAMS for various stages of floral development. Total RNA (5 μg per sample) from developing flowers was subjected to electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA fragments. (A) The expression pattern of TCS, CHS, PAL, and SAMS; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA. (B) Flower mass for various stages of development. Stages 1, 2, and 3 are flower buds, and stage 4 is in full bloom. (C) The relative amount of hybridized signal in northern blot analysis with FLA-2000.

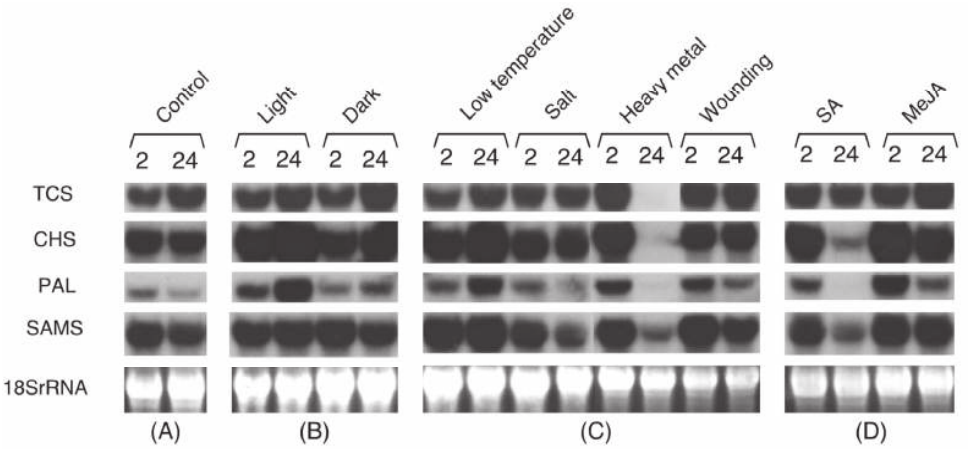

Fig. 4. Northern blot analysis of the expression of TCS, CHS, PAL, and SAMS in response to various environmental stresses and plant hormone treatments. Total RNA (5 μg each) isolated from leaves after 2 or 24 h of treatment was subjected to electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA fragments; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA. (A) Control. (B) Effect of light on gene expression. (C) Effect of environmental stress on gene expression. (D) Effect of plant hormone treatment on gene expression.

CHS, but suppressed the expression of CHS 24 h after treatment initiation. SA also suppressed the expression of PAL and SAMS 24 h after initiation of treatment. On the other hand, MeJA induced the accumulation of CHS and PAL transcripts.

Characterization of caffeine synthase gene expression in response to drought stress

Fig. 5 shows the suppression of TCS gene expression 24 h after initiation of treatment by drought stress. No effect of ABA treatment on TCS gene expression was observed. To investigate the relationship between dehydration and TCS gene expression, an analysis of the positive control gene expression was needed. Late embryogenesis abundant (LEA) proteins are members of a large group of hydrophilic glycine-rich proteins that are found in plants and are known collectively as hydrophilins; these are expressed preferentially in response to dehydration or hyperosmotic stress. In particular, dehydrins are known to be induced typically in maturing seeds or vegetative tissues following dehydration (Close, 1996, 1997). Dehydrins have a highly conserved Lys-rich 15-amino acid sequence motif referred to as the K segment, which is usually located in the C-terminus (Close, 1996, 1997). However, no dehydrin genes have been isolated from *C. sinensis*. We isolated a partial dehydrin gene that could be used for monitoring the condition of leaves of tea in response to drought stress. A PCR primer for 3'-RACE was designed on the basis of the K segment amino acid sequence. A partial putative dehydrin gene (*Csdhy1*) was isolated by RT-PCR from the total RNA extracted from young leaves. *Csdhy1* is 318 bp long and encodes the C-terminus 25 amino acid sequence (accession number AB454377).

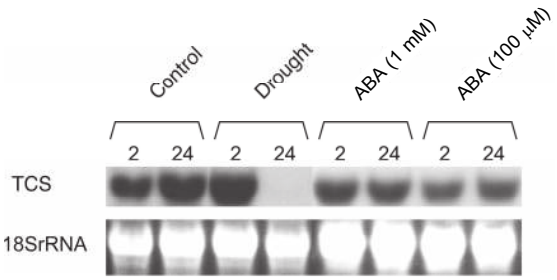

Fig. 5. Northern blot analysis of the expression of TCS in response to drought stress and ABA treatments. Total RNA (5 μg each) isolated from leaves after 2 or 24 h of treatment was subjected to electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA fragments; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA.

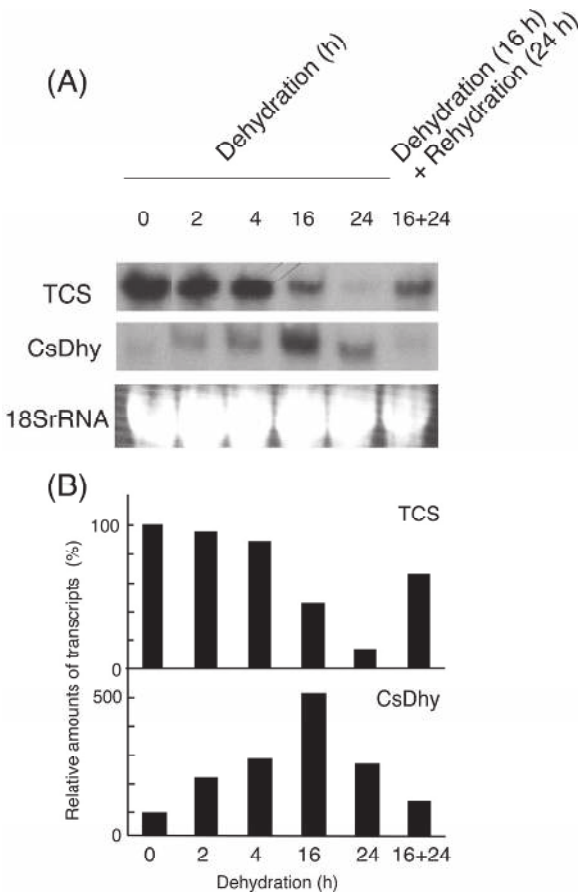

Fig. 6. Northern blot analysis of the expression of TCS in response to dehydration and rehydration. Total RNA (5 μg each) samples isolated from leaves at the indicated times (h) after dehydration or rehydration were subjected to electrophoresis on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled cDNA fragments. (A) The expression pattern of TCS; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA. (B) The relative amount of hybridized signal in northern blot analysis with FLA-2000.

The expression of *Csdhy1* was markedly induced within 2 h of dehydration, and the mRNA level increased six-fold until 16 h of dehydration (Fig. 6). Furthermore, transfer of dehydrated leaves to water after 16 h caused a decrease in the levels of the *Csdhy1* transcript that had previously been induced by dehydration, confirming that *Csdhy1* gene expression is an appropriate measure of the condition of the plants during drought stress; however, the appearance of plants subjected to 24 h of drought stress did not recover after rehydration. This observation corresponded to the decrease in *Csdhy1* transcript after 24 h of dehydration. In contrast, the expression pattern of TCS mirrored that of the *Csdhy1* transcript. Therefore, TCS expression was shown to be negatively regulated by dehydration, and transcripts of TCS accumulated again on rehydration after 16 h of dehydration.

Effects of the nitrogen source on caffeine synthase gene expression

Fig. 7 shows the accumulation of TCS transcripts 2 h after the supply of various sources of nitrogen. TCS transcripts after the supply of methionine, glutamic acid, or $NaNO_3$ were detected at levels twice those observed in control leaves. The expression level in the NH_4Cl-treated leaves was slightly higher than in the control leaves; the same result was obtained in urea-treated leaves (data not shown).

Discussion

Gene expression in response to aging

The developing leaves derived from plants were examined for changes in the accumulation of transcripts of TCS. The youngest leaves (stage 1) were not fully developed, and the leaves were unfolded after stage 2. A correlation between leaf development and the fresh weight of a leaf was observed from stage 1 to stage 4 (Fig. 2). Levels of TCS gene expression at stage 1 were the highest among all the developing stages, and fell by around 90% when the development had finished (stage 5). Fujimori *et al.* (1991) reported that caffeine synthase activity was present at high levels in very young developing leaves of tea, but absent in fully developed leaves. Our results suggest that the transcriptional regulation of TCS plays an important role in the control of caffeine biosynthesis. The difference in transcription levels between young leaves and mature leaves was remarkable; in contrast, the difference between mature leaves and old leaves was imperceptible. The expression pattern of three NMTs involved in caffeine biosynthesis in young, mature, and old leaves of the coffee plant (*Coffea arabica)* was consistent with the pattern observed in leaves of tea (Mizuno *et al.*, 2003a). Moreover, CS transcripts are easily detectable on northern blot analysis of young

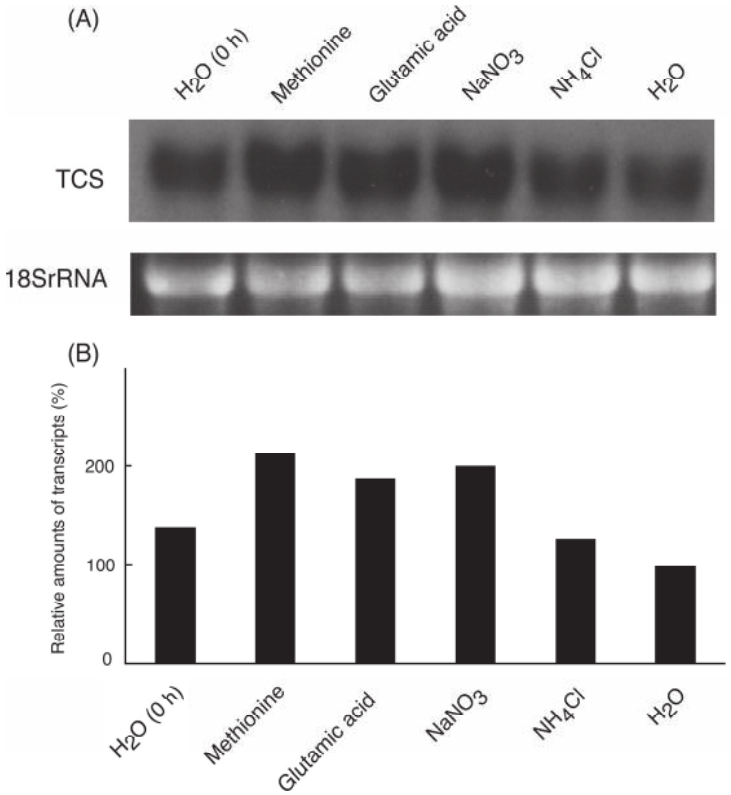

Fig. 7. Northern blot analysis of the expression of TCS in leaves after supplies of nitrogen sources. (A) The expression pattern of TCS; the bottom row shows ribosomal RNA stained with ethidium bromide to verify the amount of RNA. (B) The relative amount of hybridized signal in northern blot analysis with FLA-2000.

leaves of cacao (*Theobroma cacao*), but almost undetectable from mature leaves of this plant (Bailey *et al.*, 2005). Transcription of CS during leaf development might be controlled by common machinery in all species.

Three methylation steps occur in caffeine biosynthesis. SAM, which is widely distributed *in vivo*, is the methyl donor to the purine base (Suzuki, 1972). SAMS catalyzes the synthesis of SAM from L-methionine and ATP. To determine if the accumulation of SAMS transcripts during leaf development was linked to the biosynthesis of SAM, we analyzed the SAMS gene expression. Expression levels of SAMS, like those of TCS, were highest in stage 1, and this high level might contribute to caffeine biosynthesis. SAMS has been reported to be present as isozymes that vary in tissue-specific localization; for example, one of these isozymes is involved in lignin biosynthesis in *Arabidopsis thaliana* (Peleman *et al.*, 1989). Three SAM genes from *C. sinensis* have been registered in the database (AB041534, AY570925, and AJ277206), and at least two of these genes could be present in the leaves of the plant, because two genes were detected in RT-PCR products. It is possible that SAM for caffeine biosynthesis is supplied through expression of a specific gene. Northern blot analysis cannot discriminate between these transcripts, and the results that we obtained are estimates of the sum of the transcripts.

Many secondary metabolites, in addition to caffeine, are present in leaves of tea. In particular, tea polyphenols, which specifically include catechins, flavanones, and phenolic acid, may account for up to 30% of the dry weight of the leaves (Pan *et al.*, 2003). PAL and CHS genes have already been isolated from leaves of tea, and three genes for CHS have been identified (Matsumoto *et al.*, 1994; Takeuchi *et al.* 1994). PAL and CHS have been shown to be individually encoded by a

multigene family in higher plants (Hahlbrock and Scheel, 1989; Logemann *et al.*, 1995). The levels of PAL and CHS gene expression also decreased during the development of leaves; however, the levels gradually reduced as compared with the level of TCS. Park *et al.* (2004) evaluated the use of subtractive cDNA library analysis as a method for isolating the genes involved in the production of polyphenols and the genes of other secondary metabolites that are relatively abundant in young leaves of tea. They analyzed 588 cDNA clones, and their results showed that about 8.7% of the clones encoded enzymes involved in the secondary metabolism, with a particularly high abundance of flavonoid-metabolism proteins (5.1%). Chen *et al.* (2005) also generated 1648 expressed sequence tags and showed that 4.8% of the putative functional genes are involved in the secondary metabolism. Both caffeine biosynthesis and flavonoid biosynthesis are considered to be highly active processes in young tissues. However, the marked change in the levels of TCS transcripts between leaves in stage 1 and those in stage 2 proves that caffeine biosynthesis is strongly dependent on leaf age.

Levels of purine alkaloids and the potential for their biosynthesis are higher in flower buds than in flowers after flowering (Fujimori and Ashihara, 1990). However, TCS gene expression remained constant during flower development (Fig. 3). Our results show that the decrease in purine alkaloid biosynthesis in the developing flower is due to a factor other than TCS gene expression. It is quite likely that the supply of SAM is reduced as a result of a decrease in the levels of SAMS transcripts.

Factors influencing the expression of the TCS gene

Light did not affect the expression of the TCS and SAMS genes, unlike the PAL and CHS genes. This result supports the view that light has no short-term effect on caffeine biosynthesis in tea leaves (Koshiishi *et al.*, 2000). Similarly, no effects of low temperature exposure or salt stress on TCS or SAMS gene expression were observed. Exposure to a heavy metal for 24 h suppressed the expression of the four genes equally. This suppression is not specific for TCS gene expression, suggesting that heavy metals could check the normal metabolism universally. No effects of wounding on TCS gene expression were observed. Aneja and Gianfagna (2001) reported that caffeine production in young cacao leaves is stimulated by wounding. However, Bailey *et al.* (2005) reported that CS gene expression in cacao leaves is not induced by mechanical wounding. Furthermore, we detected no change in TCS or SAMS transcripts and a decrease in CHS and PAL transcripts on MeJA treatment (Fig. 4); furthermore, MeJA treatment for 0–24 h did not alter The TCS expression in either detached or attached leaves (data not shown). In contrast, Bailey *et al.* (2005) reported that MeJA induced CS gene expression in young red cacao leaves. The regulation of CS gene expression appears to be different in the leaves of tea and cacao.

We could not identify any environmental or chemical inducer that is involved in TCS gene expression. Analysis of the 5'-untranslated region of the TCS gene will provide important information that may permit the elucidation of the regulatory mechanism for TCS gene expression.

Effect of drought stress on TCS gene expression

We have shown that TCS transcripts disappeared 24 h after dehydration. We also examined the expression of the *Csdhy1* gene to monitor the effects of drought stress. TCS appeared to be a rehydration-inducible gene. We obtained the same results with leaves of coffee (*Coffea arabica*) under drought stress (data not shown). Suppression of CS gene expression under drought stress is not considered to be a specific response of the tea plant. Plant responses to dehydration, including gene expression, appear to involve both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 2000). The lack of an effect of ABA on TCS gene expression suggests that an ABA-independent pathway is involved in expression of the TCS gene. Oono *et al.* (2003) analyzed rehydration-inducible genes using an *Arabidopsis* full-length cDNA microarray, and they identified 152 genes. These rehydration-inducible genes were classified into three major groups, namely regulatory proteins, functional proteins involved in the recovery process from dehydration-induced damage, and functional proteins involved in plant growth. Functional proteins involved in secondary metabolism have not yet been identified as products of rehydration-inducible genes. One of the physiological roles of caf-

feine is in chemical defense to protect young soft tissues from pathogens and herbivores (Ashihara *et al.*, 2008). Developed leaves of tea are coated with wax crystals and cutin layers, and become hard. Stimulation of biosynthesis of leaf wax under drought stress has been observed in maize and tobacco (Premachandra *et al.*, 1991; Kimberly *et al.*, 2006). Either chemical or physical defense is believed to be functional in the developing leaf stage of tea, and further research is necessary to clarify this relationship.

Caffeine biosynthesis and the supply of nitrogen source

NH_4^+ and NO_3^- are the most important sources of inorganic nitrogen in soils that are readily available to plants. There is some evidence that growth of tea plants is improved by nutrition with NH_4^+ as compared with NO_3^- (Ishigaki, 1974). On the other hand, poor plant growth with NO_3^- has been reported to be largely associated with inefficient absorption of the nitrogen source (Ruan *et al.*, 2007). The similar effects of NO_3^- and glutamic acid have been interpreted in terms of an increase in the content of glutamic acid in NO_3^--supplied leaves (Ruan *et al.*, 2007). Microarray analyses of the nitrate-induced genes have been reported for *Arabidopsis thaliana* and tomato (Wang *et al.*, 2000, 2001, 2003). No genes involved in the secondary metabolism were detected as nitrate-induced genes by this analysis. Expression of the gene for phosphoethanolamine *N*-methyltransferase, which is involved in the biosynthesis of phosphorylcholine, was induced by nitrate in *A. thaliana*; however, there is a low homology between this gene and the NMTs involved in caffeine biosynthesis (Wang *et al.*, 2000). Alkaloids may be considered as nitrogen-storage products that accumulate under adverse environmental conditions. Fritz *et al.* (2006) demonstrated that nitrogen deficiency leads to a marked shift from accumulation of the alkaloid nicotine (which contains nitrogen) to accumulation of carbon-rich phenylpropanoids. They also provided further evidence that the spectrum and levels of secondary metabolites in tobacco are regulated in response to the carbon and nitrogen status. Nicotine biosynthesis appeared to respond to changes in the downstream nitrogen metabolism, rather than NO_3^-. It is quite likely that the caffeine biosynthesis is also regulated by changes in the downstream nitrogen metabolism. Further studies are needed to clarify the relationship between caffeine biosynthesis and nitrogen metabolism.

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