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FLAVONOID BIOSYNTHESIS IN TEA (*Camellia sinensis)*

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ABSTRACT

Tea leaf contains very high level of polyphenols, mostly flavonoids, which distinguishes it from other plants. These flavonoids and their oxidative products formed during fermentation and drying, largely determine the color and taste of black tea. We studied the flavonoid content of various clones of tea and found it to correlate with chalcone synthase activity in the tea leaf. Induction of chalcone synthase (CHS) activity in the tea leaf was observed during the second flush, a period where flavonoids content of tea is highest in Assam. Expressions of enzymes viz. Cinnamyl alcohol dehydrogenase (CAD), cinnamyl CoA reductase (CCR) and Peroxidase (POD) involved in the synthesis of lignin were depressed during the second flush. These observations suggest that CHS may be a key enzyme involved in catechin biogenesis in tea leaf.

Key words: Camellis sinensis, flavonoids, chalcone synthase

Abbreviations:

4CL, 4-Coumaryl Ligase; C4H, Cinnamate 4-hydroxylase; CHS, Chalcone synthase; C3H, 4-coumarate-3-hydroxylase ; CCR, Cinnamyl CoA reductase ; CAD, Cinnamyl alcohol dehrdrogenase; CHI , Chalcone isomerase ;POD, Peroxidase; DHFR, Dihydro flavonol reductase ; LAR, Leucoanthocyanidin reducatse ; CIA , Choloform : Isoamyl alcohol.

INTRODUCTION

Tea is one of the popular beverages consumed widely all over the world. It is generally believed that the polyphenolic compounds i.e. flavonoids, of tea are responsible for its useful pharmacological properties (Dixon et al., 1999;Lu et al., 2003). Raw material and the processing conditions largely influence the flavonoid content and the quality of tea (Balentine et al., 1997). The quality of the raw material in turn depends upon the agronomic practices including plucking standards, climatic, geographical and environmental conditions (Yamanishi, 1995). In addition, the distinct flavors of the various teas are due to the characteristic flavonols present in them (Peterson et al., 2004). Hence it is imperative to understand the regulation of the flavonoid biosynthesis in tea.

Flavonoids are a diverse group of plant natural products synthesized from phenyl propanoid

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and acetate derived precursors. They play an important role in growth, development and defense against microorganisms and pests (Dixon et al., 1999). , and are involved in production of phytoalexins; act as UV protectants, insect repellents and signal molecules in plant-microbe interactions (Winkel et al., 2001). Besides, they also function as complex, polymeric constituents of the leaf surface and support structures such as suberin, lignin, and other cell-wall components (Dixon et al., 1999).

Vascular plants have the unique ability to divert large amounts of carbon from aromatic amino acid metabolism into biosynthesis of natural products particularly through the phenylpropanoid pathway (Winkel et al., 1999). Phenylpropanoids are derived from cinnamic acid. The enzyme phenylalanine ammonia lyase (PAL) catalyses the deamination of phenylalanine to cinnamic acid, which is further, modified by hydroxylase and o-methyltransferases (Dixon et al., 1999). The enzyme 4-Coumaryl CoA ligase (4CL) catalyses the formation of CoA esters of cinnamic acid and these activated intermediates are used in the biosynthesis of diverse compounds via specific branch pathways, such as those leading to either flavonoid or lignin biosynthesis (Winkel, 2001).

Chalcone synthase (CHS) is pivotal for biosynthesis of flavonoids in plants (Winkel, 2001). Three full-length cDNA's (CHS 1, CHS 2, CHS 3) encoding chalcone synthase have been isolated from young leaves of Camellia sinensis and each of the cDNA encodes 389 amino acid residues, with ~ 93-96% identity to one another (Atsuko et al., 1994).

Data published on biogenesis of flavonols in tea (Punyasiri et al., 2004) showed combined DHFR/ LAR activity responsible for catechins and gallocatechins synthesis. The paper described biosynthesis of the most prominent flavonoids in tea, by considering both the enzyme activities and substrate specificities of the preceding reactions. However, the contribution of CHS, a key enzyme, of the flavonoid pathway has not been highlighted in these studies.

Advances have been made in understanding the regulation of flavonoid biosynthesis, particularly by the use of molecular genetic approaches. A number of novel regulatory proteins are filling in the void between signals that induce the pathway and well-known flavonoid regulators (Winkel et al., 2001). In this paper, we describe the findings on correlation of CHS with the catechin biogenesis in the tea leaf. The insight obtained creates an understanding of the reasons for higher flavonoid content in the second flush teas.

MATERIALS AND METHODS

Plant Material

Fresh leaves of UPASI-3, SA6 and Assamica variety were obtained from Daverashola / Thiashola, Plantations in Tamil Nadu, South India. Freeze-dried leaves of clones SA, TV-1, S3A3 of second flush were obtained from Doom-Dooma Plantations, Assam, North India. The plucking standard was 3 leaves and a bud for all the tea leaf samples.

CHS enzyme assay

A modified method for CHS enzyme assay was based on a protocol reported earlier (Klnobloch et al., 1977). Fresh tea leaves (0.5 g) were ground to a fine powder in the presence of liquid nitrogen using a mortar and pestle, then extracted using 12 ml of an extraction buffer of the following composition: 10% w/v PVPP, 700 mM potassium phosphate buffer, pH 8.0 (containing 1.5% w/v PEG, 400 mM Sucrose, 1 mM CaCl2 and 0.1% BSA) and was stirred on ice for 5 minutes. This was followed by centrifugation at 37,000 x g for 30 min at 40C. The clear supernatant thus obtained was stirred on Dowex (1x2), chloride (CL-) form for 30 min

to remove residual phenolics. On completion of stirring, the extract was filtered and subjected to centrifugation at 20,000 x g for 20 minutes. The clear supernatant obtained, termed the crude CHS extract and was stabilized using 200 mM ascorbic acid, 50 mM EDTA, 50 mM cysteine and 5 mM diethyldithiocarbamate. The assay mixture in a total volume of 1 ml consisted of buffer (0.2 M potassium phosphate, pH 7.5 containing 1.5% BSA) in the presence of crude CHS extract, 0.2 mM coumaryl CoA and 0.1 mM malonyl CoA (Sigma) that was incubated at 300C for 30 min. The reaction was stopped with 0.1N HCl and the reaction products were extracted in ethyl acetate. Product was injected into HPLC (Hypersil column C-18, RP, 100 x 4.60 mm, 3m) and naringenin chalcone was quantified. The mobile phase consisted of methanol, water, phosphoric acid (128:136:1, v/v/v) at a flow rate of 0.5 ml / min with detector maintained at 292 nm (Ebel et al., 1974)

Analysis of Catechins

Green tea leaves (2.4 g), with a plucking standard of 3 leaves and a bud was obtained from the first and second flush. Leaves were infused in 100 ml of 50% ethanol by incubating at 70oC water bath for 20 minutes with frequent stirring. The infusion was filtered into a 100 ml volumetric flask and the volume was made up with distilled water to 100 ml. The sample was filtered using 0.45mm (HATF filters) and 20ml injected into the HPLC (Bonda-Pak C-18, 60A, 4 mm, 3.9 x150 mm) for catechin estimation (Finger et al., 1992; Kang et al., 2000). Mobile Phase consisted of 20% DMF, 1% methanol and 0.5% acetic acid made up to 1 liter with water at a flow rate of 1 ml / min. The detector was a UV-Visible, l max maintained at 280 nm. The catechins epigallocatechin (EGC), epicatechin (EC), epigallocatechingallate (EGCG), catechin, and epicatechingallate (ECG) were quantified.

Northern Analysis

The RNA was extracted from the tea leaves comprising of 3 leaves and a bud obtained from first and second flush respectively as per the protocol published earlier (Jaiprakash et al., 2003). Total RNA (approximately 30mg) was run on a 1% agarose formaldehyde denaturing gel at 20V for 4 hours. Formaldehyde was washed off the gel before setting it up for the northern analysis. Northern blotting and subsequent probing and detection were performed as previously described (Fredrick et al., 1993;Jaiprakash et al., 2003).

Microarray Analysis

The Arabidopsis DNA microarray slides were procured from HHMI biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine. The slide had a total of 9,216 Expression Sequence Tags (EST's) spotted on it. Each slide contains a duplicate copy of the entire array, each of which consists of 16 sub-arrays with 24 rows and columns. The negative control genes like green fluorescent protein, globin, luciferase, phospho transferase, B-cell receptor, insulin like growth factor, tyrosin phosphatase and PHI-X-individual fragment, were printed on top of subarrays 1 and 2. In brief, the method involved extracting RNA from 2gms of MB380 leaf samples (1st and 2nd flush) which was followed by cDNA synthesis using biotin and fluorescein reaction using standard protocols. The labelled cDNA's were purified with isopropanol precipitation. cDNA synthesis was followed by cDNA analysis to ensure appropriate labelling of biotin and Fluorescein using streptavidin and anti-fluorescein. After ensuring the labelling, hybridisation was done at 65^{O}C in a humid environment for 17 hours. Several washes were given in SSC to remove unreacted probe material and finally detection was done using cyanine-3 tyramide and cyanine-5-tyramnide solution.

RESULTS AND DISCUSSION

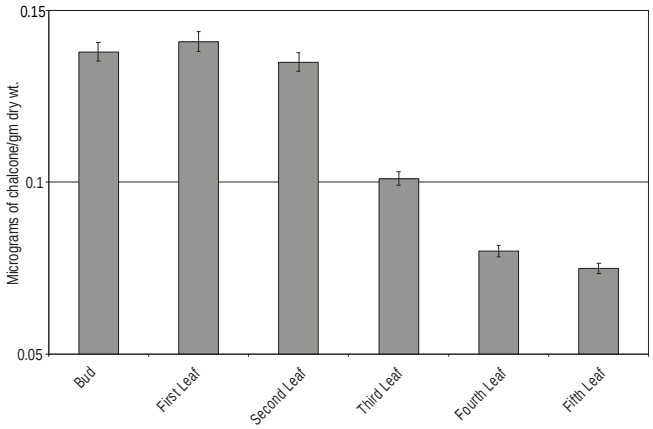
Chalcone synthase activity and catechin content

Chalcone and catechin content was found to be significantly higher in young/ juvenile leaves as compared to the mature leaves (Figure 1a/ 1b).

Figure 1

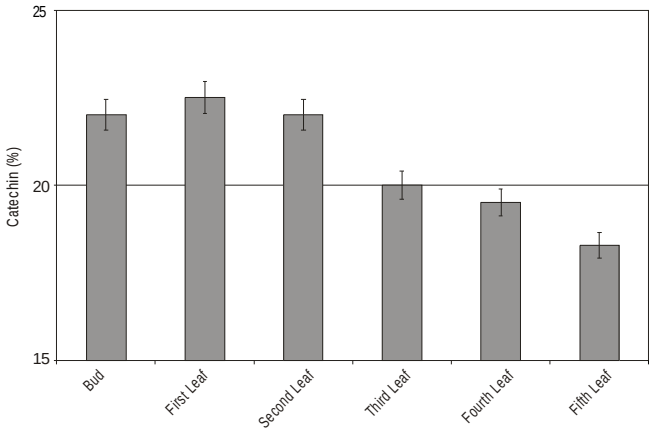
Catechin and chalcone distribution in the tea shoot in UPASI-3 clonal leaf

a) Chalcone



Chalcone synthase activity (mg of chalcone formed) in the bud to the fifth leaf in UPASI-3 leaf. CHS activity is higher in younger leaves compared to mature leaves.

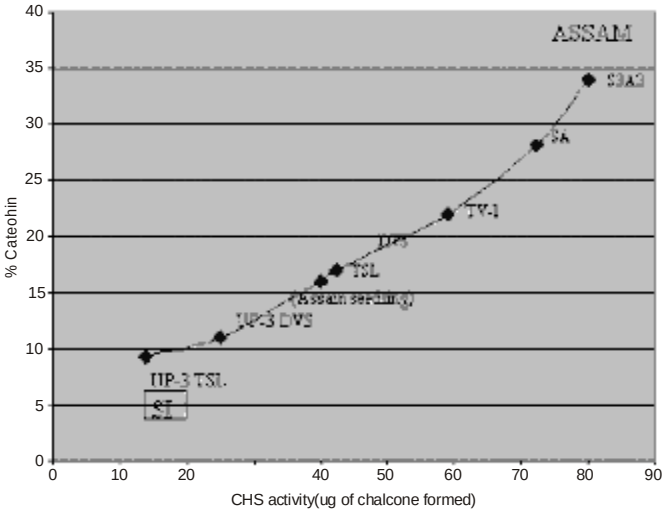
b) Catechin



Catechin distribution in the tea shoot of UPASI-3 clone. Young/ Juvenile leaves contain higher catechin compared to the older/ mature leaves.

Chalcone is a precursor to catechin biogenesis and hence a positive correlation between CHS activity and the catechin content is not surprising. This association was validated by measuring the catechin and CHS activity in tea samples spanning across different regions in India (Figure 2). Upon analysis the teas could be categorized into three main varieties as low, medium and high catechin containing leaf. The catechin content in these tea samples was directly proportional to the chalcone content in the leaf. The Assam clonal leaf (S3A3, Figure 2), with the highest catechin content also recorded the highest CHS activity, while UP-3 of South India with lowest catechin showed lowest CHS activity. The data suggest strong association between CHS activity and catechin content in the tea leaf.

Figure 2. Catechin content vs. CHS activity in leaf samples



Graph indicating CHS activity (as mg of chalcone formed) vs. the catechin content in the clones from the South Indian [4 clones] and Assam Plantations [3 clones]. Catechin content varies with samples of different geographies. It is dependent on the chalcone synthase activity of the tea leaf.

Microarray Analysis

Microarray is a powerful tool that is used to understand global changes in gene expression. Gene expression data can elucidate the mechanisms of the biological processes by identifying genes into functional pathways. In the absence of a tea (Camellia) array we have used an Arabidopsis genome (microarray) to estimate the gene expression profile in tea. Cross species hybridization has been reported in other systems. (John et al., 2005; Carmiya

et al., 2006). Genomic DNA hybridization studies were performed between Arabidopsis and tea which revealed about 70% homology in the expression profile (Venkatesh et al., our unpublished data). To the best of our knowledge, this is the first report on cross-species hybridization (CSH) between Arabidopsis and tea. Second flush (May-June) tea is known to contain elevated levels of flavonoids and volatiles (Baruah et al., 2003; Hazarika et al., 1984). This study was conducted to unravel the uniqueness associated with second flush tea and also to study the differential gene expression profiles between the first (March-April) and second flush teas. The gene profiling studies discussed in this paper was done using a tea leaf, i.e. MB380, plucked in the first and second flush respectively.

Data analysis revealed several up and down regulated genes upon comparison of the first flush with the second flush tea. The discussion on gene expression data has been restricted to only those gene(s) involved with flavonoid biogenesis in the two flushes. CHS, the key enzyme associated with the flavonoid pathway was up regulated while Cinnamte-4Hydroxylase (C4H) was down regulated (Table 1).

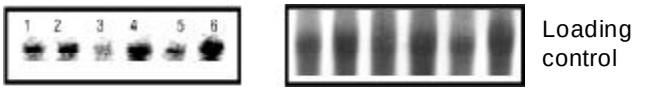
Table 1 Microarray analysis
Key enzymes involved in flavonoid and lignin biosynthesis

Functional Classification	Gene expression	Gene expression
	(Up)	(Down)
Phenylpropanoid & flavonoid pathway	Chalcone synthase (CHS)	Cinnamte-4-hydroxyalse(C4H)
Lignin Pathway	Caffeoyl -O- methyl transferase	Cinnamyl Alcohol Dehydrogenase
		Cinnamoyl COA reductase
		Anionic peroxidase

C4H is a cytochrome P450 dependent enzyme that catalyzes the first oxygenation step of the general phenylpropanoid metabolism in higher plants. The compounds formed are essential for lignification and defense against predators and pathogens (Teutsch et al.,1993).

On the other hand CAD, CCR and POD, important enzymes associated with the lignin pathway (Kawasaki et al. 2006; Braz Ramos et al. 2001) were down - regulated in the second flush. Alteration of CAD expression in plants is reported to have an effect on lignin composition, while reduction in levels of CCR has been directly associated with a decreased lignin content (Leple et al., 1998). Anionic peroxidase involved in polymerization of the monolignols (Lagmini et al., 1987) was also down regulated in the second flush. Decreased expression in majority of the key genes associated with lignin pathway would also impact lignin content in the second flush. Northern analysis was performed to validate the CHS expression by using a labeled CHS probe. The CHS signals were higher in the second flush tea (Figure 3a) and also correlated positively with catechin content in the second flush, further corroborating the data obtained using micro array studies (Figure 3b). (Editor's note: Some referees felt that microarray studies is not really useful/relevant because Arabidopsis microarray has been used instead of tea microarray.) A positive correlation between catechin content and the CHS activity was observed. Our results confirmed that second flush tea leaves contain higher levels of flavonoids (catechins) when compared to the first flush. These studies also suggests that the

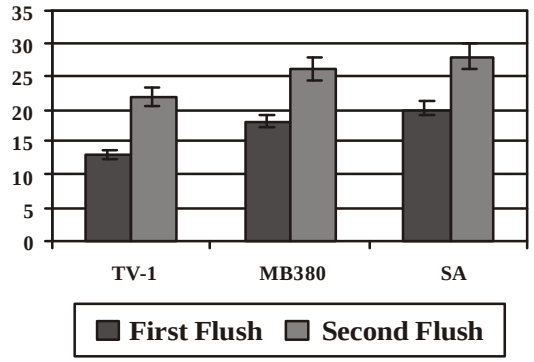
Figure 3 Northern analysis and catechin content
a) Northern using CHS3 as probe
1: TV1 first Flush,2: TV1 secondFlush,,3: MB380 first Flush 4: MB380 second Flush,5: SA first Flush,6: SA second Flush



a) RNA northern analysis detected using CHS 3 as a probe. Lanes corresponding to second flush (TV-1, MB380, SA) demonstrates enhanced CHS expression compared to first flush.

second flush tea leaves accumulates flavonoids by down regulating the key enzymes involved in the lignin biosynthesis and thus redirecting all the carbon flow towards synthesis

b) Catechin content in first vs. second flush



b) Catechin content in the first vs. second flush tea leaf samples (TV-1, MB380, SA) have been compared. Higher catechins were observed in the second flush. Catechin data is an average of 3 independent sampling trials (n=3)

of flavonoids.

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