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Investigation of the Site-Specific Accumulation of Catechins in the Tea Plant (*Camellia sinensis* (L.) O. Kuntze) via Vanillin−HCl Staining

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Histochemical staining using vanillin−HCl is a potential tool to identify the site-specific accumulation of catechins in the tea plant (*Camellia sinensis* (L.) O. Kuntze). Using this technique revealed that catechins existed ubiquitously in all inspected tissues in young tea leaf, but the distribution was concentrated in the vascular bundle and palisade tissue, whereas the large parenchyma cells of the main vein contained lower amounts of catechins. At the subcellular level, catechins were located mainly in the chloroplasts of mesophyll cells and in the vessel wall. In young stems, catechins could be detected in most cells except the parenchyma cells of the pith and the cortex, whereas, in roots, catechins could be detected only in those cells surrounding the pericycle. Moreover, differing distributions of catechins were found in calluses cultivated in darkness and light. On the basis of HPLC analyses, six main types of catechins were present in tea leaves, stems, calluses, and chloroplasts; however, roots contained only epicatechin.

KEYWORDS: ***Camellia sinensis*****; catechins; histochemical localization; vanillin−HCl reagent; staining; HPLC analysis**

INTRODUCTION

As the most important secondary metabolites in the tea plant (*Camellia sinensis* (L.) O. Kuntze), catechins (flavan-3-ol) account for up to 30% of the dry weight of fresh tea leaves (*1*) and are the dominant flavor compounds in tea beverage. Numerous studies have revealed that catechins have antioxidant activity and promote osteoblastic activity (*2*). In addition, they are a class of promising naturally occurring phytochemicals with antihypertensive effects, antibacterial and antiviral activities, and antifibrotic properties, and they inhibit enterovirus function (*1*, *3*).

The biosynthetic pathway of nongalloylated catechins (including (+)-catechin, (−)-epicatechin, (+)-gallocatechin, and (−)-epigallocatechin) has been well addressed (*4*−*6*). However, little is known about the biosynthesis of galloylated catechins (including (−)-epigallocatechin-3-gallate and (−)-epicatechin-3-gallate) and the localization of both nongalloylated and galloylated catechins in the tea plant. Information about the localization of catechins in the tea plant would be helpful for understanding how catechin biosynthesis is controlled and would provide additional insights into this metabolic pathway.

Flavonoids can accumulate in various intracellular regions, including both smooth and rough endoplasmic reticulum, plastids, the Golgi body, vacuoles, cell walls, and even the nucleus (*7*−*15*). Of these, vacuoles may be the predominant organelle for catechin accumulation (*12*), whereas flavanols also were detected in nuclei in tea flower buds (*C. sinensis* L.) (*15*). Until now, the localization of catechins in the tea plant has not been well studied.

Various staining reagents, such as $FeSO_4$, $FeCl_3$, vanillin−HCl, the Hoepfner−Vorsatz stain, and 4-dimethylaminocinnamaldehyde (DMACA), have been used to detect phenolic substances (*12*, *15*−*18*). For example, vanillin−HCl reagent and DMACA can react with flavan-3,4-diol, flavan-3-ol, and proanthocyanidins (oligomers or polymers of flavan-3-ols and flavan-3,4-diol units) (*17*−*20*).

In this study, we found that vanillin−HCl staining was a sensitive and simple method for the histochemical localization of catechins in the tea plant. We reveal here the site-specific accumulation of catechins in various tissues and organs of the tea plant determined by means of vanillin−HCl staining.

MATERIALS AND METHODS

Plant Materials. Young roots, stems, and leaves of the tea plant (*C. sinensis* (L.) O. Kuntze) were collected from the tea garden of Anhui Agricultural University, China. Tea callus was cultivated from the young shoot of the tea plant (*C. sinensis* (L.) O. Kuntze) on B_5 medium (*21*). Callus was subcultured every 21 days in 100 mL Erlenmeyer flasks containing 30 mL of solid medium in the dark for over 1 year. Callus subcultured under light (2000 lx) for 21 days formed a green callus.

Chemicals. (+)-Catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate were purchased from Shanghai RongHe Pharmaceutical Co., Ltd. Dihydroquercetin, dihydromyricetin, kaempferol, quercetin, and myricetin were obtained from Sigma Co., Ltd. Tea saponin was a product of Hangzhou Zhongye Natural Plant Technology Co., Ltd. Other chemicals were of the highest grade available.

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Specificity of Vanillin−HCl Reagent. The various flavonoid compounds (including catechins, flavanonols, and flavonols) were dissolved in 95% ethanol, and tea saponin was dissolved in water. To test the specificity of the vanillin−HCl reagent, 1 mL of flavonoid or saponin solution of various concentrations was added to 5 mL of 1% (w/v) vanillin−HCl reagent. For catechins, the reaction mixture was assayed immediately at 500 nm using a spectrophotometer (2800 UV−vis, UNICO Instruments Co., Ltd. Shanghai, China). For flavanonols, flavonols, and saponin the mixture was assayed after 20 min, again at 500 nm. The specificity of the vanillin−HCl reagent was determined using the slopes of reaction curves with the different compounds.

Tissue Slicing and Staining. To slice tea tissues, fresh carrot was used as a supporter. Fresh carrot without xylem was cut into a 5 cm × 0.5 cm × 0.5 cm section. A seam was cut longitudinally at the end of the section as an adaptor for tea organ or callus. The supporter containing tea organ or callus was sliced by free-hand sectioning. Samples for observation were prepared by standard freehand sectioning (*22*) and observed under a microscope (XQT-2, COIC). Images of the section were recorded before and after staining.

The section was stained with 1% (w/v) vanillin−HCl reagent. Sufficient reagent was added to one side of the section and absorbed into it with tissue paper on the opposite side of the section for about 5 min, by which time the section was well stained and the excess reagent on the surface of the section was completely removed.

To observe the accumulation of catechins in the nucleus, the section was first stained with 0.01 mol/mL iodine solution and then with 1% (w/v) vanillin−HCl reagent. To observe the accumulation of anthocyanidin and leucoanthocyanidin in tea plant cells, the section was stained with HCl reagent without vanillin.

Catechin Extraction. To extract catechins from various tissues and organs of the tea plant, 1 g of tea sample (young leaf, stem, phloem-removed stem, and root) or 3 g of callus was crushed under liquid N_2 and macerated with 10 mL of 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. The water solution was extracted three times with ethyl acetate. The organic phase was evaporated and dissolved in 1 mL of methanol. Catechins in the methanolic solution were separated by HPLC or TLC.

To extract catechins from chloroplasts, 50 g of fresh tea leaf was homogenized for 10 s in 100 mL of extraction buffer (0.33 M sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 4 mM cysteine, and 5 mM vitamin C, pH adjusted to 8.0 with KOH). The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The precipitate was resuspended in 30 mL of extraction buffer, placed on a sucrose gradient (30%/45%/60%, 30 mL), and centrifuged at 30000*g* for 30 min at 4 °C. A green stripe between 45 and 60% on the sucrose gradient was collected and washed three times with precooled washing buffer (0.33 M sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 5 mM vitamin C, pH adjusted to 8.0 with KOH) and then centrifuged at 2000*g* for 10 min at 4 °C to separate intact chloroplasts. One milliliter of the precipitate, containing the intact chloroplasts, was dissolved in 10 mL of 95% ethanol. The ethanol solution was assayed at 652 nm using a spectrophotometer and the chlorophyll content calculated according to the formula $C = A_{652nm} \times 1000/34.5$ (mg/mL). To examine the catechins present in the chloroplasts, the ethanol solution was evaporated, dissolved in hot water, and extracted three times with ethyl acetate. The organic phase was concentrated, dried, and redissolved in 1 mL of methanol. The catechins present in the methanolic solution were determined by HPLC.

Catechin Analysis. To analyze catechins in the above-described methanol extracts by HPLC, all samples were filtered through a 0.22 μm membrane and then separated on a Phenomenex Synergi 4u Fusion-RP80 column (250 × 4.6 mm) with detection at 280 nm 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). Following the injection of 5 μL of sample, a linear gradient was initiated at a flow rate of 1.2 mL/min: B from 10 to 13% over 20 min, then from 13 to 30% between 20 and 40 min, and from 30 to 10% between 40 and 41 min. Peaks were identified by retention time compared with catechin standards.

To analyze catechins in the above-described methanolic solution by TLC, the methanol solution was spotted on silica GF254 TLC sheets (5 × 20 cm). TLC sheets were developed in trichloromethane/methanol/formic acid (28:10:1, v/v) and then sprayed with 1% (w/v) vanillin−HCl reagent. The spots of catechins were identified by their R_f value, and color was compared with catechin standards.

Table 1. Responses of Selected Flavonoid and Saponin Compounds to 1% (w/v) Vanillin−HCl

class	compound	color	standard curve[a]
catechins	C	brick red	$y = 27.833x - 0.036$
	EC	brick red	$y = 20.443x + 0.013$
	GC	brick red	$y = 14.462x + 0.048$
	EGC	brick red	$y = 18.262x + 0.028$
	ECG	magenta	$y = 19.580x - 0.029$
	EGCG	magenta	$y = 11.865x + 0.019$
flavanonols	DHQ	orange-red	$y = 0.413x + 0.069$
	DHM	orange-red	$y = 0.139x + 0.096$
flavonols	K	orange	$y = 0.113x - 0.002$
	Q	orange	$y = 0.375x + 0.005$
	M	orange	$y = 0.818x + 0.002$
saponin	S	orange-red	$y = 0.079x + 0.030$

[a] *y* units are OD; *x* units are mg/mL.

RESULTS

Specificity of Vanillin−HCl Reagent Reacting with Catechins. In the 1970s, the specificity of vanillin reacting with flavanols was investigated by testing 15 flavonoids and 2 chromone compounds for reaction with vanillin−HCl (*23*). Sarkar et al. (*23*) showed that vanillin−HCl is useful for the detection and quantitative analysis of flavanols in plants but that the possibility of interference by dihydrochalcones and anthocyanins should be considered. In the present study, 12 compounds, including catechins, flavanonol, flavonol, and saponin, were examined to test the specificity of vanillin−HCl reagent for catechins in the tea plant. The specificity of the vanillin−HCl reaction was identified by the slopes of reaction curves with various compounds (**Table 1**). The flavanonol, flavonol, and saponin could react with the vanillin−HCl reagent; however, their reaction slopes were 1−2 orders of magnitude lower than those of the catechins. Reaction of catechins with the vanillin−HCl reagent is both sensitive and fast (Figure S1A, B). Furthermore, the quantities of flavanonols, flavonols, flavanones, and saponin in the tea plant are much lower than that of catechins (*24−26*). Finally, TLC and HPLC analyses also confirmed that the major components in tea leaf that were reacting with the vanillin−HCl reagent were catechins (Figures S1C,D and S2A).

Although anthocyanidin and leucoanthocyanidin display a red color in acidic solution, which could interfere with the localization of catechins, our experiment showed no staining occurred after immersion of tea leaf in acidic solution without vanillin (Figure S2B,C), suggesting that the potential interference caused by anthocyanidin and leucoanthocyanidin could be ignored.

Other factors influencing vanillin−HCl reagent staining, including the solution concentration, staining time, and sample fixing, were further investigated. The staining results suggested that the vanillin concentration in the acidic solution should not be below 1% (w/v) (Figure S2D−F). The section could be observed with a microscope immediately after staining or within the first 30 min to avoid fading of the red color (Figure S2G−I). Materials to be tested should not be fixed with FAA solution (formaldehyde/acetic acid/ethanol = 1:1:18, v/v) before vanillin−HCl staining, as this causes poor distinguishability due to red color dispersion or even a lack of staining in the xylem (Figure S2J,K). When unfixed material was stained with the vanillin−HCl reagent, the cells

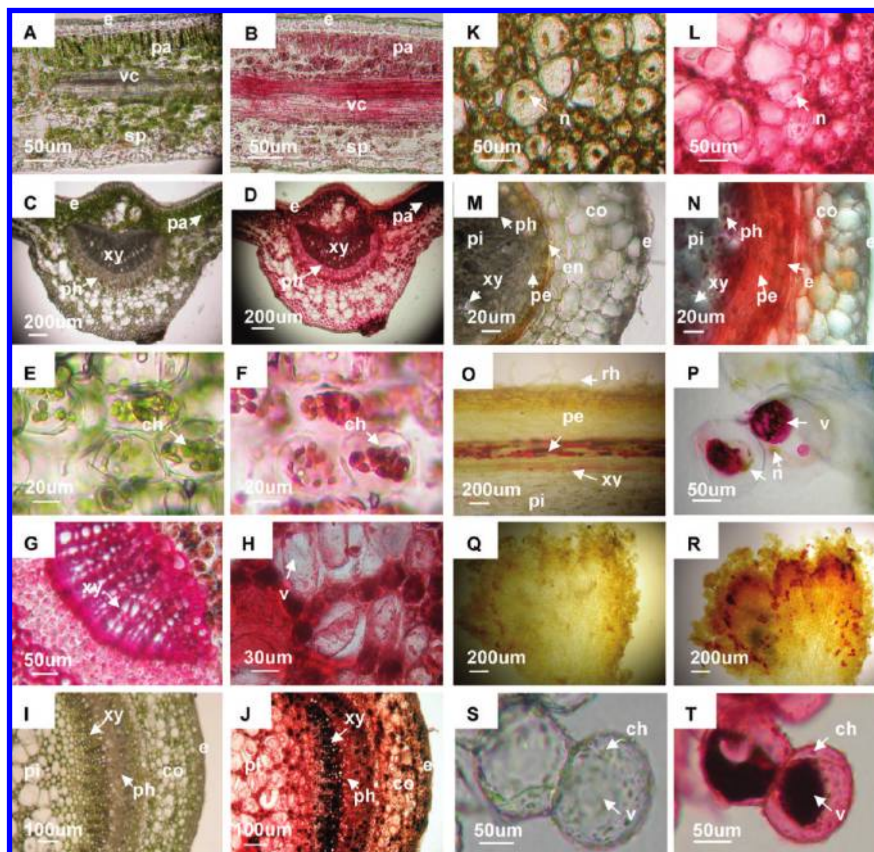

Figure 1. Histochemical localization of catechins in different tea organs and tea callus. Red represents catechin-accumulating areas after 1% (w/v) vanillin−HCl staining: (**A** and **B**, **C** and **D**, **E** and **F**) transverse leaf sections before and after staining; (**G**) transverse main vein section after staining; (**H**) parenchyma cells of pith from young stem after staining; (**I** and **J**) transverse young stem section before and after staining; (**K**) parenchyma cells of pith from young stem after staining with 0.01 mol/mL iodine solution; (**L**) parenchyma cells of pith from young stem after double-staining; (**M** and **N**) transverse young root section before and after staining; (**O**) longitudinal root section grown from tea calluses after staining; (**P**) callus cultivated under darkness after double-staining; (**Q** and **R**) callus cultivated under darkness before and after staining; (**S** and **T**) green callus cultivated under light before and after staining. e, epidermis; pa, palisade parenchyma; vc, vascular cylinder; sp, spongy; co, cortex; xy, xylem; ph, phloem; pe, pericycle; en, endodermis; ch, chloroplast; rh, root hair; pi, pith; v, vacuole; n, nucleus.

suffered slight plasmolysis, which was actually beneficial for observing the staining of cell membranes and cell walls (**Figure 1L**,**P**). Initial staining for the nucleus with iodine solution did not influence subsequent vanillin−HCl staining (**Figure 1L**).

Taken together, these results indicate that vanillin−HCl staining is a sensitive, simple, and relatively specific method for the histochemical localization of catechins in tea plant samples.

Catechin Accumulation in Different Tea Organs. To avoid the loss of cell contents, the sections of young tea leaf, stem, root, and callus were sliced via free-hand sectioning and immediately stained with the vanillin−HCl reagent. Using this method, we obtained sharp microphotographs (**Figure 1**). After vanillin−HCl staining, the red-stained catechins in fresh tea leaf could be detected easily in almost all tissues, especially the dark red seen in palisade parenchyma cells, vascular bundles, chloroplasts of mesophyll cells, and vessel walls and the pale red seen in the epidermis, although no detectable staining was seen in the large parenchyma cells of the main vein (**Figure 1A**−**G**). These results suggested that catechins were present at different levels in different cellular and subcellular locations in fresh tea leaf.

In the same way, significant staining differences were observed in the epidermis, cortex, vasculature, and pith of young stems (**Figure 1I**,**J**). Deeper staining was found in green collenchyma cells in the cortex, small green cells in the pith, and central cylinder cells, whereas lighter staining was observed in large parenchyma cells in the cortex and pith (**Figure 1J**). The xylem in the central cylinder was stained the deepest red. No detectable staining was observed in the vacuoles of large parenchyma cells in the pith (**Figure 1H**). The accumulation of catechins in the nuclei of large parenchyma cells in the pith was observed by double staining with iodine solution and the vanillin−HCl reagent (**Figure 1K**,**L**).

The main compartments of tea root that accumulated catechins were around the pericycle, whereas no catechin accumulation was observed in the cortex or xylem vessels (**Figure 1M**,**N**). As the same catechin accumulation pattern was observed in root grown from callus (**Figure 1O**), we deduced that tea root had the ability to synthesize and accumulate catechins.

In the outer cells of callus cultivated in darkness, staining was observed in vacuoles (**Figure 1Q**,**R**). This result suggested that catechins were accumulated mainly in the vacuole (**Figure 1P**). However, in green callus cultivated under light, catechins were observed in chloroplasts and the cytoplasm as well as in vacuoles (**Figure 1S**,**T**).

Content of Catechins in Different Tea Organs. The amounts of different catechins in tea plant samples were determined using HPLC analysis (**Table 2**; **Figure 2**). The results showed that the total amount of catechins was the highest in the tea leaf, followed by the stem, and there was only a small amount of catechins present in the root. The quantity of catechins present in callus tissue was far less than that in fresh tea leaves and young stems.

The relative concentrations of individual catechins were fairly consistent among fresh tea leaf, young stem, stem xylem without

cortices, callus, and leaf cell chloroplasts (**Figure 2A**,**B**,**D−F**), but particular catechins varied greatly at certain locations; for example, there was a higher proportion of epicatechin in stem xylem without cortices. Only epicatechin was detected in young tea roots (**Figure 2C**).

DISCUSSION

In acid solution, vanillin reacts with the flavonoid ring at the 6- or 8-position to give a red-colored compound. Sarkar et al. (*23*) found that a single bond between C-2 and C-3 and free meta-oriented hydroxyl groups on the B ring were the essential structural requirements for this reaction and that flavanols and dihydrochalcones showed a higher sensitivity to vanillin−HCl stain. Naringenin and flavanonol produced less color than the flavanols due to the electron-withdrawing effect of the carbonyl group at C-4. A pink or red color in acidic solution is given by anthocyanins and leucoanthocyanidin. Thus, some interfering substances should be avoided when the vanillin−HCl test is used for the quantitative determination of flavanols or proanthocyanidins (PAs) in plants (*27*, *28*). In the tea plant, the quantities of PAs, anthocyanidin, leucoanthocyanidin, and flavonols are much lower than that of catechins (*24−26*, *29*) so the vanillin−hydrochloric acid stain is widely used for rapid and simple quantitative measurement of catechins in tea (*30*, *31*). Furthermore, the vanillin−hydrochloric acid stain has also been used for the histochemical localization of phenols (*32−34*). Our experimental results indicate that the flavanonol, flavonol, and saponin could react with the vanillin−HCl reagent; however, their reaction slopes were 1−2 orders of magnitude lower than those of the catechins, and the results also show that the potential interference from anthocyanidin and leucoanthocyanidin could be ignored as compared with catechins. Therefore, the vanillin−HCl method is a useful tool for the specific histochemical localization of catechins in tea plant samples. With the procedure of vanillin−HCl staining for catechins, we found that free-hand sectioning could easily result in high-quality images.

Many experiments have indicated that flavonoids accumulate in numerous intracellular compartments, such as smooth and rough endoplasmic reticulum, plastids, the Golgi body, vacuoles, the cell wall, and even the nucleus (*7−15*). However, the biosynthesis of flavonoids had long been believed to take place exclusively in the endoplasmic reticulum followed by transfer, primarily to

Table 2. Catechin Content (Milligrams per Gram of Dry Weight) of Different Tea Organs and Tea Callus

organ/callus	GC	EGC	C	EC	EGCG	ECG	sum
leaf	1.09	2.04	3.87	0.99	55.19	7.08	70.26
young stem	0.54	2.56	0.68	8.65	23.54	5.76	41.73
stem xylem	0.04	0.22	0.08	2.09	0.36	0.60	3.39
root				0.59			0.59
callus	0.03	0.10	0.09	0.19	1.39	0.59	2.39
chloroplast (mg/mg of chl)	0.01	0.01	0.02	0.03	0.22	0.11	0.40

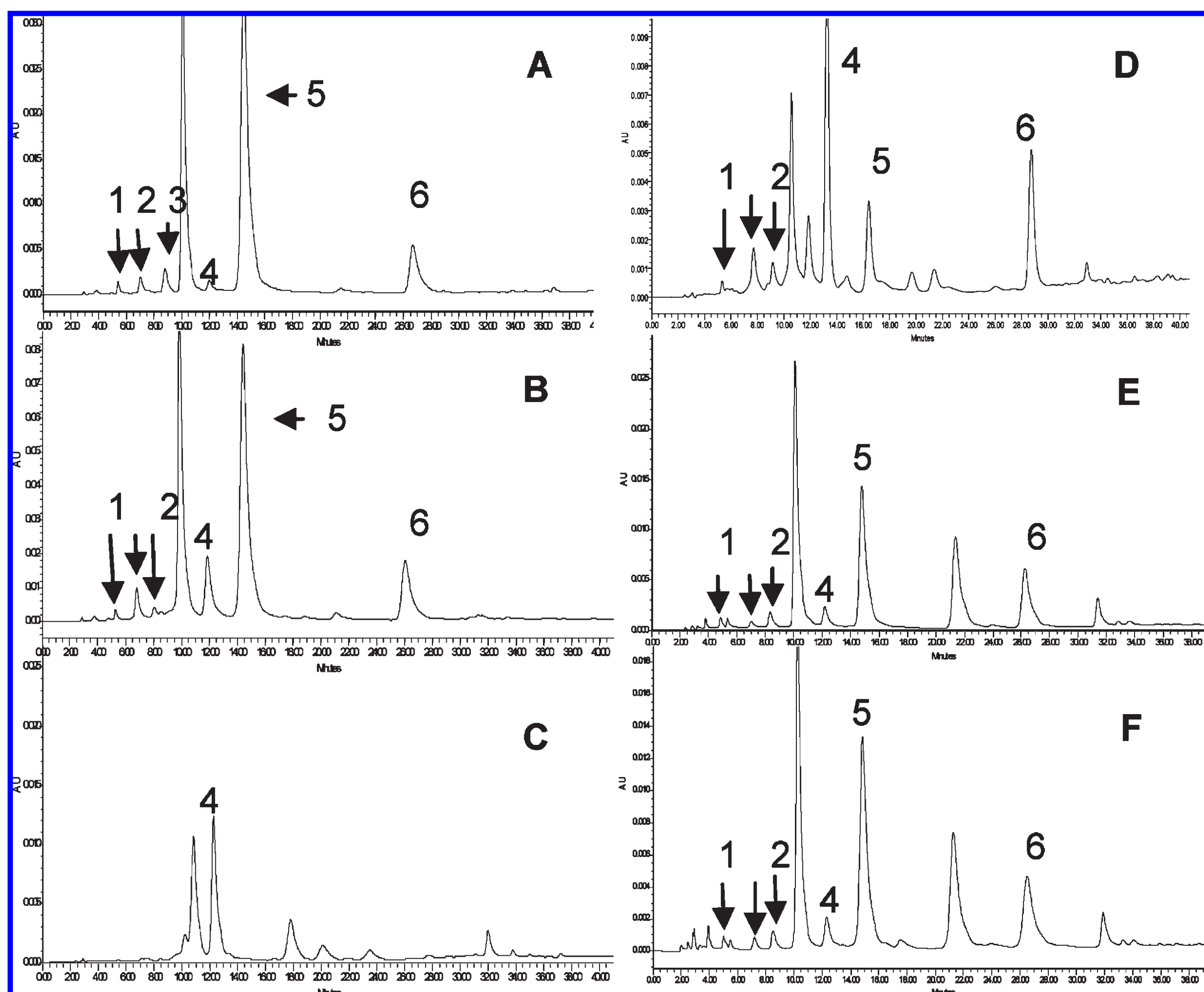

Figure 2. Chromatograms of HPLC analysis of catechins from different tea organs and tea callus: (**A**) leaf; (**B**) stem; (**C**) root; (**D**) stem xylem; (**E**) chloroplast; (**F**) callus. Peaks: 1, GC; 2, EGC; 3, C; 4, EC; 5, EGCG; 6, ECG.

vacuoles, by processes involving multidrug resistance-associated protein or multidrug and toxic compound extrusion proteins (*35*, *36*). Some plants, such as sorghum (*37*), transfer flavonoids via small vesicles. Apart from the endoplasmic reticulum, Kefeli et al. (*38*) deduced that chloroplasts were a site for phenol synthesis on the basis of experiments with chloroplasts of willow leaves (*Salix* spp.). Saslowsky et al. (*14*) found that two key enzymes, chalcone isomerase and chalcone synthase, exist in the nucleus in several cell types in *Arabidopsis*, and thus they assumed that flavonoid biosynthesis occurs not only in the cytoplasm but also in the nuclei of some cells. They also proposed that flavonoids play a role in protecting DNA from UV and oxidative stress damage. It has been reported that chloroplast-localized flavonoids can act as scavengers of $^1O_2$ (*39*). The results of our present work show that chloroplasts are one of the main sites for catechin accumulation.

Recent studies have been focused on the internal mechanism of phenol transfer from the synthetic site (cytoplasm) to the effective site (vacuole, cell wall). In petunia and soybean and in the maize aleurone layer, the transfer of pigments to the vacuole requires glutathione *S*-transferase and an ATP-dependent glutathione pump of the ABC family (*11*, *40*). Debeaujon et al. (*41*) found that in *Arabidopsis* testa, the transfer of proanthocyanidin precursors to the vacuoles involved a subtransport protein belonging to the multidrug and toxic compound extrusion family. In addition to intracellular movement, Buer et al. (*42*) observed the long-range transport of flavonoids via DPBA fluorescence photomicrography in *Arabidopsis* mutants that could not synthesize phenolic substances. They showed that the long-range transport from stem to root of naringenin and dihydroflavonol, the intermediates of the phenolic biosynthetic pathway, required the participation of ABC family proteins to provide cell-to-cell transport. Our experiments found that catechins accumulate in vascular bundles of young tea leaf and stem, but in roots, catechins accumulate only around the pericycle and were not observed in the xylem. Therefore, the transport of catechins from stem to root cannot be excluded. Interestingly, catechins accumulated in the same places in root grown from callus and natural tea root, which illustrated that tea root has the capacity to synthesize catechins.

Although catechin accumulation was not observed in the vacuoles of tea leaf and young stem, catechins were found to concentrate in vacuoles of the callus, suggesting that the accumulation of catechins in the callus was different from that in fresh tea leaf or young stem. Further studies are required to reveal the mechanisms of the synthesis, accumulation, and transfer of catechins in the tea plant.

ABBREVIATIONS USED

ABC, ATP-binding cassette; C, (+)-catechin; DHM, dihydromyricetin; DHQ, dihydroquercitin; DMACA, 4-dimethylaminocinnamaldehyde; DNA, deoxyribosenucleic acid; DPBA, diphenylboric acid-2-aminoethyl ester; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; EDTA, ethylenediaminetetraacetic acid; FAA, formalin−aceto−alcohol; GC, (+)-gallocatechin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; K, kaempferol; M, myricetin; Q, quercetin; S, tea saponin; TLC, thin-layer chromatography; UV, ultraviolet (light); PAs, proanthocyanidins.

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Supporting Information Available: Figures S1 and S2 showing data obtained during optimization of the staining protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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