Condensed Tannin Composition Analysis in Persimmon (Diospyros kaki Thunb.) Fruit by Acid Catalysis in the Presence of Excess Phloroglucinol

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Persimmon (Diospyros kaki Thunb.) accumulates soluble condensed tannin (CT) in fruit which is responsible for its astringency trait. In this study, we analyzed the CT composition in persimmon fruit with phloroglucinol, and identified and characterized novel epigallocatechin-3-O-gallate-phloroglucinol (EGCG-P) adducts as one of the main CT components of persimmon fruit. Analysis with phloroglucinol in persimmon cultivars revealed the different tendencies of the CT composition and component ratio among the four astringency types (PCNA, PVNA, PVA, and PCA), which are categorized by their patterns of astringency loss. The concentration of the main CT component in persimmon fruit, epigallocatechin-3-O-gallate (EGCG), was particularly different among astringency types. Further analysis of the fruit at various maturation stages will help in understanding the different mechanisms of CT accumulation among astringent types. Our results demonstrated that the phloroglucinol methodology is useful for CT composition analysis in persimmon fruit and will contribute to future studies on the astringency trait in this fruit.

Key Words: condensed tannin, Diospyros kaki, epigallocatechin-3-O-gallate, phloroglucinol.

Introduction

Condensed tannin (CT) (also called proanthocyanidin) is a phenolic oligomer resulting from the polymerization of flavan-3-ol units, which consists of two types of subunits, extension and terminal units (Fig. 1A, Aron and Kennedy, 2008; Dixon et al., 2005) and is synthesized via the flavonoid pathway (Dixon et al., 2005; Lepiniec et al., 2006). Flavonoids generally provide protective functions in plants, particularly against herbivores and UV irradiation (McMahon et al., 2000; Winkel-Shirley, 2001). They also act as antioxidants with beneficial effects on human health, including protection against free radicals, and cardiovascular and metabolic diseases (Aron and Kennedy, 2008; Cos et al., 2004). As a final product of the flavonoid pathway, CT also contributes to the quality of many important plant products, such as wine, tea, cocoa and some berries (Aron and Kennedy, 2008).

Japanese persimmon (Diospyros kaki Thunb.) accumulates soluble CT in fruit, which is responsible for its strong astringency trait, during the early stages of fruit development. Matsuo and Ito (1978) analyzed the toluene-α-thiol degradation products of persimmon fruit CTs isolated from ‘Hiratanenashi’ (pollination variant astringency; PVA type). The results suggested that the proposed CT structure consists of two types of flavan-3-ols; catechin (C) and gallocatechin (GC), and their gallate forms; CA-3-O-gallate (CG) and GC-3-O-gallate (GCG), with a molar ratio of 1:2:1:2, respectively. These units are linked by the C-4 position through C-8; however, this characterization does not refer to the 2,3-cis/trans configuration of flavan-3-ols in persimmon fruit (Fig. 1A, catechin vs. epicatechin and gallocatechin vs. epigallocatechin). The study by Tanaka et al. (1994) is the only study that distinguished this cis/trans configuration of flavan-3-ols by HPLC analysis following thiolysis. This situation is mainly due to technical difficulties in CT composition analysis.

Acid-catalyzed cleavage with benzyl mercaptan or phloroglucinol has been the major method of CT composition analysis (Fig. 1B for methodology, Scofield et al., 2001). Although previous reports suggested that benzyl mercaptan is the preferred reagent, an unpleasant odor that occurs during thiolysis makes it difficult to use...
However, the problem of low efficiency in the reaction with phloroglucinol has been reported by Scofield et al. (2001). A recent study by Kennedy and Jones (2001) clearly justified the use of excess phloroglucinol in acid-catalyzed cleavage studies of CT in grapevine (*Vitis vinifera*). CT composition analyses in apple (*Malus × domestica*), *Arabidopsis thaliana* and other plant species using the same method showed that acid-catalyzed cleavage with excess phloroglucinol is a convenient and effective method (Pang et al., 2007; Pourcel et al., 2005; Takos et al., 2006).

In this study, we applied the same method using phloroglucinol to analyze CT composition in persimmon fruit. In preliminary analyses with phloroglucinol, we detected three peaks for flavan-3-ols phloroglucinol adduct products.

**Fig. 1.** Representative condensed tannin (CT) structure (A) and methodology for CT composition analysis with phloroglucinol or thiophenol (B). (A) Each CT component is shown with their structure depending on the groups at the 5' position of the B-ring (R1), 3 position of the C-ring (R2), and 2,3-cis/trans configuration of the C-ring. (B) When phloroglucinol (left) or thiophenol (right) undergoes nucleophilic addition to the 4 position, we can detect extension units as phloroglucinol or thiophenol adducts, respectively, while terminal units as monomers.

**Fig. 2.** HPLC profile for ‘Yokono’ phloroglucinol adduct analysis.
products; epicatechin-phloroglucinol (EC-P), epigallocatechin-phloroglucinol (EGC-P) and epicatechin-3-O-gallate-phloroglucinol (ECG-P), and one for an unidentified major peak (Fig. 2). Persimmons are classified into four types according to the pattern of astringency-loss on the tree; (i) pollination-constant non-astringent (PCNA), (ii) pollination-variant non-astringent (PVNA), (iii) pollination-variant astringent (PVA) and (iv) pollination-constant astringent (PCA) (Yonemori et al., 2000). We initially tried to identify this uncharacterized major peak by acid catalysis with phloroglucinol and to quantify the phloroglucinol adducts among the four types of persimmon cultivars. We also aimed to reveal the different tendencies of the CT composition and component ratios among these astringency types. Our approaches using phloroglucinol disclosed that the CT composition in persimmon fruit and its analysis may provide new insight into CT polymerization mechanisms, which are now sought in the field of plant science (Xie and Dixon, 2005), because the degree of polymerization in persimmon fruit is considerably higher than that in other plant species (Aron and Kennedy, 2008; Matsuo and Itoo, 1978).

Materials and Methods

Plant materials

Immature ‘Yokono’ (PCA) fruits were sampled in the experimental orchard of Kyoto University, Kyoto, Japan, on July 2, 2008 to isolate and characterize the unidentified major peak in HPLC analysis with phloroglucinol (Fig. 2). Immature ‘Mikado’ (PCNA), ‘Sangoku-ichi’, ‘Niikura’ (PVNA), ‘Akadzu’, ‘Hiratanenashi’ (PVA), ‘Yokono’, and ‘Miyazaki-tanenashi’ (PCA) fruits were sampled in the same orchard for CT composition analyses among these astringency types. ‘Jiro’ (PCNA) fruits were sampled in the Experimental Farm of Kyoto University, Takatsuki, Osaka, Japan. Three fruits per each cultivar were sampled on June 19, 2007. The fruit mesocarp was diced (approximately 0.5 × 0.7 × 1.0 cm) and lyophilized into a freeze-dried sample.

Isolation and characterization of an unidentified major phloroglucinol adduct in persimmon fruit

Freeze-dried (0.995 g) ‘Yokono’ fruit powder was added directly to 10 mL phloroglucinol reagent (500 mg phloroglucinol and 100 mg ascorbic acid in 1% HCl/MeOH; v/v/v) and incubated at 50°C for 20 min. This reaction was stopped using 10 mL of 200 mM sodium acetate. After concentrating the aqueous solution, it was extracted with ethyl acetate three times and evaporated under reduced pressure at 35°C. The extract was dissolved in 800 μL MeOH, of which 200 μL was spotted on a reverse-phase thin layer chromatography (TLC) plate and developed with 0.2% aqueous acetic acid to separate the phloroglucinol adducts from excess phloroglucinol. The separated component was collected with TLC, dissolved with 100% MeOH and filtered through a 0.45 μm filter. After concentrating the filtrate under reduced pressure at 35°C, a crude and unidentified component was separated again by TLC developed with toluene:acetone:formic acid (6:6:1; v/v/v), according to a previous report by Merghem et al. (2004). The isolated component was lyophilized and characterized by 1H-NMR Varian Inova 300 (300 MHz; Varian Inc., Palo Alto, USA), 2D NMR (H-H COSY), and MALDI-TOF MS Bruker Reflux III (Bruker Corporation, Yokohama, Japan) according to the previous report by Zhang and Lin (2008).

The 1H-NMR spectra were recorded on a Varian INOVA 300 spectrometer in acetone-d6/methanol-d4 (1/1, v/v) with tetramethylsilane as an internal standard, by using pulse sequences for one- and two-dimensional spectra. Chemical shifts (δ) and coupling constants (J) are given in δ-values (ppm) and Hz, respectively. MALDI-TOF MS spectra were recorded with Bruker MALDI-TOF MS REFLEX III. For ionization, a nitrogen laser was used. All spectra were measured in the reflector mode using external calibration. Compounds were measured with 2,5-dihydroxybenzoic acid (DHB) as a matrix.

Procedure for determining the CT composition in persimmon fruit

To extract CTs, freeze-dried samples were ground to a fine powder and 10 mg was extracted in 1 mL of 70% acetone with 0.1% ascorbic acid for 24 h in the dark. The samples were then centrifuged, and each of the 200 μL supernatant aliquots were transferred to a new tube and dried under vacuum at 30°C for 60 min. One of the two tubes was used to analyze free monomers of flavan-3-ols and the other was used for acid-catalyzed cleavage of the CT in the presence of excess phloroglucinol by the method mentioned in a previous report by Downey et al. (2003). These reactions were stopped with 200 mM sodium acetate (twice the volume of the phloroglucinol reagent) and then a vanillin solution was added (1 mg vanillin in 5 mL 1% HCl/MeOH; v/v), as the internal standard for subsequent HPLC analysis.

Samples were subjected to reverse-phase HPLC LC2010 (Shimadzu, Tokyo, Japan) using a Wakosil-II 5C18 RS (5 μm, 250 mm × 4.6 mm) analytical column protected by a guard column containing the same material. Elution was performed with 0.2% aqueous acetic acid (solvent A) (v/v) and MeOH (solvent B) using an elution program: 1% B for 30 min, a gradient to 15.5% B for 35 min and gradient to 45% B for 35 min, followed by washing with 100% B for 15 min and a return to the initial conditions (1% B). Analysis was performed at 30°C with a flow rate of 1 mL min⁻¹ and detection at 280 nm. For the LC-MS analysis, we used an LCMS-2010A (Shimadzu) with a Shim-pack VP-ODS (5 μm, 150 mm × 2 mm).

Concentrations of free monomer and hydrolysed terminal subunits were determined by comparing them...
Results and Discussion

Identification of the CT components in persimmon fruit with phloroglucinol

HPLC analysis of five flavan-3-ols and flavan-3-ols gallates phloroglucinol adducts (C-P, EC-P, GC-P, EGC-P, and ECG-P) has been reported in grapevine (Kennedy and Jones, 2001) and pea (Merghem et al., 2004). Based on these reports, we preliminarily analyzed persimmon fruit CTs following acid catalysis in the presence of excess phloroglucinol under the same HPLC conditions but found that some peaks overlapped under these conditions. Hence, we performed the analysis under different conditions (see Materials and Methods) according to the report by Downey et al. (2003), which has been used in other CT studies with phloroglucinol (Bogs et al., 2005; Takos et al., 2006). Using this condition, we detected clearly separated peaks (Fig. 2). HPLC analysis of persimmon ('Yokono'), grapevine ('Delaware') and pea ('Sanren'), and LC/MS analysis of 'Yokono' allowed us to identify EC-P, EGC-P and ECG-P adducts; however, C-P and GC-P adducts were not detected in the persimmon fruit. This result indicates that, except for C and GC the others are contained as extension units of the persimmon fruit CT. Apart from the EC-P, EGC-P, and ECG-P peaks, we detected an unidentified major peak immediately following the EC-P peak (see Fig. 2, indicated by arrow).

As the major peak from ‘Yokono’ of HPLC, this unidentified major compound was isolated by the TLC method. MALDI-TOF MS analysis of the isolated product using 2,5-dihydroxybenzoic acid (DHB) as a matrix revealed that the molecular weight m/z 605.23 was detected as a pseudo ion peak of sodium adduct. Namely, the molecule weight of the compound is m/z 582. Furthermore, the proton NMR spectrum of the main product revealed that the coupling constant (J2,3) of the major compound was approx. 1 Hz, recorded in acetone-d6/methanol-d4 (1/1, v/v). The C2 and C3 protons appeared at δ 5.37 (broad s) and 5.25 ppm (dd, J = 1.8 and 1.4 Hz), respectively. On the other hand, Kennedy and Jones (2001) reported that C2 and C3 protons of (-)-epicatechin-3-O-gallate-(4β→2)-phloroglucinol appeared at δ 5.45 (d) and 5.23 ppm (dd) with J2,3 < 1 Hz, respectively, recorded in acetone-d6. Davis et al. (1996) reported that C2 and C3 protons of epigallocatechin-3-O-gallate appeared at δ 5.07 and 5.56 ppm with J2,3 = 1.4 Hz, respectively, recorded in acetone-d6; however, the coupling constant (J = 6.7 Hz) between H-2 and H-3 of (+)-catechin indicates 2,3-trans configuration (Cai et al., 1991). These studies proved that the main product is epigallocatechin-3-O-gallate-(4β→2)-phloroglucinol (EGCG-P) with 2,3-cis configuration. Moreover, the coupling constant (about J = 0 Hz) between H-3 and H4 of epigallocatechin 3-O-gallate-
Characterization of the CT composition in persimmon cultivars

In phloroglucinol analyses of CTs in persimmon fruit, we mainly detected the formation of phloroglucoligal adducts for CT extension units but few hydrolyzed monomers for the terminal CT units were also found (data not shown). This indicates a high degree of CT polymerization in persimmon fruit, which is consistent with a previous report by Matsuo and Ito (1978). We detected four 2,3-cis-flavanols and 3-ols (i.e., EC, EGC, and their gallate-ester ECG and EGCG) as CT extension units in persimmon fruit (Fig. 2). Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are involved in the biosynthetic pathways of 2,3-cis- and 2,3-trans-flavanols, respectively (Dixon et al., 2005; Tanner et al., 2003; Xie et al., 2003, 2004). Therefore, the high ratio of 2,3-cis-flavanols in the persimmon fruit is presumable due to the considerably higher transcript level of ANR than that of LAR, as suggested in reports by Akagi et al. (2009a, b) and Nakagawa et al. (2008).

On comparing the persimmon cultivars, we could not detect differences in the CT compositions among the four astringency types (P > 0.05), although EGCG concentration was likely lower in the order of PCNA, PVNA, PVA, and PCA cultivars (Table 1). Previous reports suggested that the CT concentration in the PCNA cultivar is markedly reduced after mid-June, whereas non-PCNA cultivars can accumulate CT constantly until August and show little reduction in the concentration of CT (Ikegami et al., 2005). Our results from samples collected on June 19 showed minimal differences in the CT composition and concentration between PCNA and the other three non-PCNA types. Thus, the CT composition in PCNA cultivars may dramatically change accompanied by a change in the CT concentration in later mature stages. In CT component ratio analysis between cultivars, it was indicated that the total ratio of C, EC, and ECG (although CG was not detected), in which the catechol nucleus constitutes the B-ring (see Fig. 1), was clearly higher in PCNA cultivars than that in the other three non-PCNA cultivars (Table 2). These tendencies are consistent with a previous report by Nakatsubo et al. (2002). Concentrations of C, EC, and ECG were slightly high in the PCNA types. Presumably, this was also due to the low EGCG concentration in PCNA, resulting in high C, ECG, and EGC ratios against total CT in PCNA cultivars (Table 1). Furthermore, the ratio of the galloyl form in the PCNA and PVNA cultivars

Table 1. CT composition in eight persimmon cultivars estimated using phloroglucinol. Each CT component concentration is shown as [mg per 10 mg DW].

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>PCNA</th>
<th>PVNA</th>
<th>PVA</th>
<th>PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Jiro'</td>
<td>33.1 ± 4.1 b[a]</td>
<td>33.9 ± 3.2 b</td>
<td>39.8 ± 3.2 b</td>
<td>11.1 ± 2.9 d</td>
</tr>
<tr>
<td>'Mikado'</td>
<td>12.8 ± 2.9 a</td>
<td>16.1 ± 2.8 a</td>
<td>6.7 ± 2.8 ab</td>
<td>12.5 ± 4.5 a</td>
</tr>
<tr>
<td>'Sangokuichi'</td>
<td>391.7 ± 42.1 a</td>
<td>382.2 ± 41.2 a</td>
<td>246.3 ± 30.8 b</td>
<td>304.8 ± 32.9 a</td>
</tr>
<tr>
<td>'Niikura'</td>
<td>706.5 ± 92.4 bc</td>
<td>632.1 ± 109.2 c</td>
<td>751.9 ± 82.3 bc</td>
<td>698.3 ± 30.3 bc</td>
</tr>
<tr>
<td>'Hiratanenashi'</td>
<td>205.8 ± 27.1 bc</td>
<td>208.7 ± 41.2 bc</td>
<td>151.7 ± 18.8 c</td>
<td>258.2 ± 37.6 ab</td>
</tr>
<tr>
<td>'Akadu'</td>
<td>645.8 ± 72.6 cd</td>
<td>902.7 ± 85.1 bc</td>
<td>1176.9 ± 130.7 bc</td>
<td>1352.0 ± 186.1 ab</td>
</tr>
<tr>
<td>'Yokono'</td>
<td>23.4 ± 4.1 b[a]</td>
<td>23.6 ± 4.2 bc</td>
<td>23.6 ± 4.2 bc</td>
<td>23.6 ± 4.2 bc</td>
</tr>
<tr>
<td>'Miyazakitanenashi'</td>
<td>130.7 ± 15.4 bc</td>
<td>1532.0 ± 42.1 bc</td>
<td>151.5 ± 22.2 c</td>
<td>203.0 ± 30.9 bc</td>
</tr>
<tr>
<td>'Sanokuranenashi'</td>
<td>150.4 ± 17.8 bc</td>
<td>1176.9 ± 42.1 bc</td>
<td>218.5 ± 51.2 bc</td>
<td>1864.1 ± 214.2 a</td>
</tr>
<tr>
<td>'Niikura'</td>
<td>186.1 ± 17.8 bc</td>
<td>201.2 ± 17.8 bc</td>
<td>118.1 ± 17.8 bc</td>
<td>225.4 ± 17.5 b</td>
</tr>
<tr>
<td>'Hiratanenashi'</td>
<td>130.4 ± 17.8 bc</td>
<td>1532.0 ± 42.1 bc</td>
<td>151.5 ± 22.2 c</td>
<td>203.0 ± 30.9 bc</td>
</tr>
<tr>
<td>'Akadu'</td>
<td>186.1 ± 17.8 bc</td>
<td>201.2 ± 17.8 bc</td>
<td>118.1 ± 17.8 bc</td>
<td>225.4 ± 17.5 b</td>
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<td>118.1 ± 17.8 bc</td>
<td>225.4 ± 17.5 b</td>
</tr>
</tbody>
</table>

Table 2. Component CT rates (%) that had a galloyl group at the 3 position of the C-ring (galloyl form) and whose B-ring contained a catechol.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>PCNA</th>
<th>PVNA</th>
<th>PVA</th>
<th>PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Jiro'</td>
<td>43.7 a</td>
<td>54.7 b</td>
<td>51.9 b</td>
<td>64.1 d</td>
</tr>
<tr>
<td>'Mikado'</td>
<td>30.6 a</td>
<td>31.4 a</td>
<td>18.5 b</td>
<td>20.2 b</td>
</tr>
<tr>
<td>'Sangokuichi'</td>
<td>57.1 bc</td>
<td>17.1 bc</td>
<td>16.7 bc</td>
<td>14.4 d</td>
</tr>
<tr>
<td>'Niikura'</td>
<td>61.7 cd</td>
<td>62.7 d</td>
<td>65.1 d</td>
<td>14.4 d</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences among cultivars at the 5% level (ANOVA followed by Tukey’s test).
was lower than that in PVA and PCA cultivars (Table 2), which was also mainly due to differences in EGCG content.

A qualitative difference in the astringency trait between PCNA and the other three non-PCNA types has been suggested (Ikeda et al., 1985). Molecular analysis of CT biosynthesis in PCNA and non-PCNA indicated that the expression levels of many structural genes in the CT biosynthetic pathway show a considerable reduction in PCNA with fruit development (Akagi et al., 2009a, b; Ikegami et al., 2005). Akagi et al. (2009b) also suggested that a reduction in the expression of DkMyb4, which is a Myb transcription factor, and the resulting down-regulation of the two structural genes, flavanone 3’5’-hydroxylase (ANR) and ANR, which are regulated by DkMyb4, are particularly responsible for the differences in the composition or component ratio of the CT in the PCNA type. However, the PVNA, PVA, and PCA types are quantitative traits that are responsible for the quantity of volatile compounds from seeds (Yonemori et al., 2000). It would be interesting to see that different tendencies of the CT composition or component ratio were observed among PVNA, PVA, and PCA in the early mature stages when seeds have not developed (Tables I and 2). In this study, we measured the CT composition and component ratio in persimmon cultivars only at one sampling point. More detailed measurements throughout fruit maturation will contribute to characterization of the astringency trait in persimmon fruit.

**Literature Cited**


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