A Micropipette Method for Collecting Vacuolar Sap of Intact Tannin and Parenchyma Cells in Persimmon

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Abstract. A method for collecting the vacuolar contents of intact tannin and parenchyma cells of persimmon (*Diospyros kaki* Thunb.) fruit using a micropipette was developed. Thin sections of the mesocarp tissue from mature persimmon fruit, 'Miyazaki-mukaku' and 'Hiratanenashi', were placed on a glass slide. Using a micromanipulator and an inverted microscope, a micropipette was inserted into a vacuole and its contents were withdrawn. A 5-nL sample of vacuolar sap was collected per tannin cell from 'Hiratanenashi' and 7 nL from 'Miyazaki-mukaku', whereas only 2 nL was withdrawn from adjacent parenchyma cells. Analyses of the vacuolar sap revealed that the tannin cells of both cultivars contained 10% to 12% (m/v) of tannin as (+)-catechin equivalents and 10% to 13% (m/v) of soluble sugars, whereas the parenchyma cells contained trace amounts of tannins and ≈20% of soluble sugars. Tannin cells contain only a slight amount of sucrose, in contrast to a relatively large amount in parenchyma cells.

The fruit of astringent-type persimmons contain a high level of tannins that are accumulated exclusively in numerous, large tannin cells or idioblasts. When such persimmons are treated to remove the astringency factor after harvest, tannins coagulate into an insoluble mass. To understand the mechanism of tannin coagulation, an investigation into the nature of tannin cells was initiated.

Manabe (1982b) isolated coagulated tannin cells from 'Saijo' fruit treated with dry ice. He measured the tannin concentration by redissolving it in boiling 2 N HCl for 30 min. Itamura and Fukushima (1989) collected coagulated tannin cells from 'Hiratanenashi' fruit treated with ethanol, carbon dioxide, or acetaldehyde, and determined some mineral components in the cell. In both reports, the researchers tried to clarify the mechanisms leading to tannin coagulation in tannin cells. However, these reports dealt with coagulated tannin cells after artificial treatment for astringency removal, whereas we tried to investigate the constituents of intact, pretreated tannin cells and adjacent parenchyma cells.

In recent years, micromanipulation has made rapid progress in plant research (Ding et al., 1992; Oparka and Prior, 1988; van Bel and van Rijen, 1994). Hashimoto et al. (1986) collected cell contents from *Macleaya cordata* (Willd.) R. Br. root and *Sanguinaria canadensis* L. rhizome using a special micro-

manipulator needle for determining alkaloid content per single cell by high-performance liquid chromatography. They called this method "histochemical chromatography." We adapted some of these techniques to investigate the contents of tannin cells by collecting cell sap from tannin and parenchyma cells separately. The details of our method for collecting sap from intact cells of persimmons and the results of our analyses of their constituents are described.

Materials and Methods

Plant source. Fruit were obtained from mature trees of astringent-type persimmons, 'Miyazaki-mukaku' and 'Hiratanenashi', growing at the experimental orchard of Kyoto Univ., at mature stages of both fruit in Oct. 1995. These fruit contained ≈1% to 1.5% of

soluble tannins as (+)-catechin equivalent and 10% to 15% total sugars on a fresh mass basis. The tissue block (10 mm in length × 7 mm in width × 5 mm in height) was taken from the equatorial region of the fruit from which 300-μm sections were prepared by a DSK-1000 microslicer (Dosaka EM, Kyoto, Japan). Then, several slices were placed on a glass slide and the slide was fixed on the stage of an inverted light microscope (Olympus IMT-2; Olympus, Tokyo, Japan).

Collection of cell sap. The micromanipulator system MMS-77 (Shimadzu Co., Kyoto, Japan) used for collecting vacuolar sap consisted of a micropipette holder connected to a micro syringe, a monitor, a video cassette recorder, and a charge coupled device camera (Fig. 1). Images were monitored and videotaped during micromanipulation. A glass micropipette was fabricated from 25-μL-volume glass capillary (Drummond Co., Broomall, Pa.) using a micropipette tension (MPT-1; Shimadzu Co., Kyoto, Japan) according to the manufacturer's instructions. Because the pore of the micropipette was too small to withdraw cell sap, the tip was damaged slightly by pushing it down on a glass slide before use.

The glass micropipette, connected to a micro syringe, was gradually brought close to a flesh section on the microscope stage, and inserted into a targeted cell at a 45° angle. Vacuolar sap was withdrawn from the cell by lowering the pressure pneumatically through the syringe. Tannin cells were distinguished from normal parenchyma cells by their large size and characteristic refraction. After collecting the vacuolar sap, the micropipette was withdrawn from the cell, removed from its holder, and placed onto the microscope stage to measure the volume by using scales displayed on the monitor. The volume of collected sap was calculated as

$$V = 1/3 \cdot \pi \cdot r^2 \cdot l \cdot 10^{-6}$$

where V = volume of collected cell sap (nL), r = radius of the glass micropipette (μm) at the meniscus of the collected sap, and l = length (μm) between the tip of the glass micropipette and the meniscus of collected sap.

Fig. 1. Diagram of micromanipulator system MMS-77 (Shimadzu Co., Kyoto, Japan) for collecting vacuolar sap of persimmon fruit cells. CCD = charge coupled device; CRT = cathode ray tube.

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After the sap volume was measured, the glass micropipette was attached to the holder again, and the contents expelled into 5 μ L of distilled water that had been placed on a glass slide covered with Parafilm (American National Can, Neenah, Wis.). Then, the drop was quantitatively transferred to a microfuge tube and stored at -20°C until analysis.

Tannin and sugar analysis. The vacuolar sap samples collected from three single tannin or parenchyma cells, respectively, were combined and the analyses for their tannin and sugar concentrations were run in triplicate. The tannin concentration was measured as total phenolics by the Folin-Denis method (Swain and Hillis, 1959) and was calculated as (+)-catechin equivalent.

For sugar analysis, combined samples were freeze-dried and silylated with trimethylsilylating reagent. The silylated sugars were analyzed on a gas-liquid chromatograph equipped with flame ionization detector and glass column (2 m $\times$ 3 mm in diameter) packed with 3% SE-52 on acid-washed and silanized Chromosorb W (Sweeley et al., 1963). The sugar concentration was expressed on a mass/volume (m/v) basis.

Results and Discussion

Using the micropipette system, an average of 6.75 nL (± 0.54 ; $n = 27$) was collected from tannin cells of 'Miyazaki-mukaku' and 5.16 nL (± 0.30 ; $n = 19$) from 'Hiratanenashi'. The respective values for the parenchyma cells were 1.82 nL (± 0.13 ; $n = 23$) from 'Miyazaki-mukaku' and 1.62 nL (± 0.16 ; $n = 20$) from 'Hiratanenashi'. Each collection of the sap was continued until just before the collapse of the vacuole. For tannin cells, however, the micropipette was pulled out at earlier stages of extraction than for parenchyma cells, since the membranes of tannin cells were more fragile and collapsed earlier than those of parenchyma cells. Thus, vacuolar sap from tannin cells was not collected completely, while that from parenchyma cells was collected almost completely. However, the vacuoles shrank during the extraction of both types of cells (Figs. 2 and 3), indicating that the contents of the targeted cells seemed to be withdrawn without contamination from adjacent cells or intercellular spaces. Additional work will be needed to show lack of contamination from other saps, such as cytoplasmic or apoplastic solution, possibly by using marker enzymes of the cytoplasm or apoplast.

The tannin concentration in tannin cells was $\approx 10\%$ to 11% (m/v) for 'Miyazaki-mukaku' and 'Hiratanenashi', whereas only a trace was detectable in the parenchyma cell (Table 1). These results support the finding in a histological study on persimmon fruit in which Yonemori (1986) reported that tannins are confined only to the vacuole of large tannin cells.

Parenchyma cells in both cultivars contained $>20\%$ (m/v) total sugars, whereas tannin cells contained $<15\%$ (m/v) (Table 1). Although tannin cells contain high levels of soluble sugars, sucrose concentration was low

in both cultivars, especially in 'Miyazaki-mukaku', compared to the high sucrose content in the parenchyma cells (Table 1). The predominant sugar was glucose in tannin cells, whereas it was sucrose in parenchyma cells. There were some differences in sugar ratios between the two cultivars. Sucrose level was extremely high compared to glucose and fructose levels in the parenchyma cells of 'Miyazaki-mukaku', but their levels were nearly the same in 'Hiratanenashi'.

Zheng and Sugiura (1990) reported that sugars in persimmon fruit consist of fructose, glucose, and sucrose, and that their ratios differed among cultivars, which they attributed to differences in acid invertase activity. A

cultivar with low acid invertase activity has a high ratio of sucrose to glucose and fructose; conversely, a cultivar with a high acid invertase activity has a low sucrose to reducing sugar ratio. Zheng (1989) reported 'Miyazaki-mukaku' as having a high ratio, whereas 'Hiratanenashi' had a low ratio. Our results on sugar composition in parenchyma cells agree with those of Zheng.

Manabe (1982a) reported that nitrogenous compounds and minerals coagulated simultaneously with tannins, making them all insoluble. However, Itamura and Fukushima (1989) reported that nitrogen in coagulated tannin was not detectable but that low amounts of minerals were found. Ittah (1993) sug-

Fig. 2. Photomicrographs of a persimmon fruit parenchyma cell during withdrawal of its contents (A to C). Note short arrows in (C) that show shrinking of the vacuole. Long arrows in (A), (B), and (C) indicate the tip of micropipette. Bars = 125 μ m.

Fig. 3. Photomicrographs of a persimmon fruit tannin cell during withdrawal of its contents (A to C). Note short arrows in (B) and (C) that show shrinking of the vacuole. Long arrows in (A), (B), and (C) indicate the tip of micropipette. Bars = 125 μ m.

Table 1. Tannin and sugar concentrations of vacuolar sap extracted from tannin and parenchyma cell in 'Miyazaki-mukaku' and 'Hiratanenashi' persimmon fruit.

Constituent	Tannin and sugar concentrations (% m/v)			
	Cultivar			
	Miyazaki-mukaku		Hiratanenashi	
	Cell type	Cell type	Cell type	Cell type
	Tannin	Parenchyma	Tannin	Parenchyma
Tannin ^a	10.20 $\pm$ 0.35 ^a	0.91 $\pm$ 0.04	10.76 $\pm$ 0.08	1.46 $\pm$ 0.23
Sucrose	0.76 $\pm$ 0.22	14.65 $\pm$ 1.18	2.33 $\pm$ 0.98	8.74 $\pm$ 0.51
Glucose	7.46 $\pm$ 0.78	2.98 $\pm$ 0.19	7.28 $\pm$ 0.79	6.32 $\pm$ 0.19
Fructose	3.63 $\pm$ 0.21	2.89 $\pm$ 0.15	4.33 $\pm$ 0.47	5.90 $\pm$ 0.56
Sucrose/reducing sugars	0.07 $\pm$ 0.01	2.36 $\pm$ 0.17	0.22 $\pm$ 0.10	0.72 $\pm$ 0.05
Glucose/fructose	2.08 $\pm$ 0.21	1.05 $\pm$ 0.12	1.74 $\pm$ 0.22	1.11 $\pm$ 0.11

^aTannin concentration was calculated as (+)-catechin equivalent.

^aAverage $\pm$ SE (n = 3).

gested that the process of astringency removal involved sugars through the formation of glycoside between the soluble tannins and glucose. As tannin cells contain relatively high levels of glucose compared with fructose (Table 1), this may be a possible mechanism in tannin coagulation.

Several methods for collecting and analyzing cell sap exist, e.g., protoplast and vacuole isolation (Komor and Thom, 1989), compartmental analysis method (Yamaki and Ino, 1992), and the aphid method for phloem sap analysis (Moing et al., 1992). By collecting cell sap from persimmon fruit with a micropipette, we found that tannin cells contained $\approx$ 11% tannin and 12% total sugars, whereas parenchyma cells contained only a trace of tannin and 20% sugars. Furthermore, this method allowed us to illustrate that the metabolism of phenolic compounds and sugars differed in tannin and parenchyma cells in the mesocarp tissue of persimmons.

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