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Single-Cell Metabolite Profiling of Stalk and Glandular Cells of Intact Trichomes with Internal Electrode Capillary Pressure Probe Electrospray Ionization Mass Spectrometry

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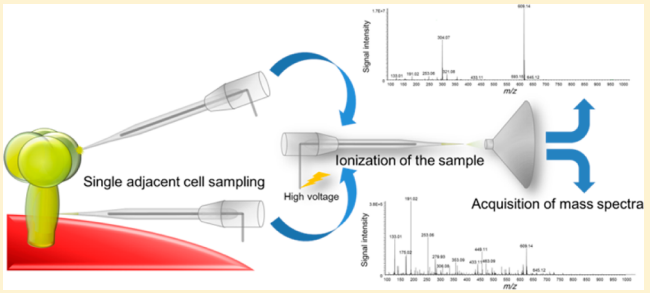
Supporting Information

ABSTRACT: In this report, we developed the pressure probe electrospray ionization-mass spectrometry with internal electrode capillary (IEC-PPESI-MS) which enables high spatial-resolution cell sampling, precise postsampling manipulation, and high detection sensitivity. Using this technique, a comparative *in situ* single-cell metabolite profiling of stalk and glandular cells, the two adjacent cell types comprising a trichome unit in tomato plants (*Solanum lycopersicum* L.), were performed to clarify the extent of metabolic differentiation between two cell types as well as among different types of trichomes. Owing to high sensitivity of the system, less than a picoliter cell sap from a single stalk cell sufficiently yielded a number of peaks of amino acids, organic acids, carbohydrates, and flavonoids. The minimal cell sap removal from a stalk cell without severe disturbance of trichome structure enabled sequential analysis of adjacent glandular cell on the same trichome, which showed the presence of striking differences in metabolite compositions between two adjacent cell types. Comparison among different types of trichome also revealed significant variations in metabolite profiles, particularly in flavonoids and acyl sugars compositions. Some metabolites were found only in specific cell types or particular trichome types. Although extensive metabolomics analysis of glandular cells of tomato trichomes has been previously documented, this is the first report describing cell-to-cell variations in metabolite compositions of stalk and glandular cells as well as in different trichome types. Further application of this technique may provide new insights into distinct metabolism in plant cells displaying variations in shape, size, function and physicochemical properties.



Understanding metabolism at single-cell resolution is a critical task in biological sciences to clarify mechanisms underlying cellular differentiation, interaction, function, and responses to environmental influences.[1−3] With increasing demands for single-cell analyses, rapid technical progresses have been made with a variety of analytical platforms including fluorescence microscopy, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry (MS), among which MS is the most widely utilized method with high detection sensitivity for metabolites in a high-throughput fashion.[1−4] Several studies have recently described MS-based single-cell shotgun metabolomics approaches in which cell sampling was often carried out with tapered microcapillaries[5−10] and etched metal needles,[11,12] followed by electrospray ionization (ESI)[6−12] or by matrix-assisted laser desorption/ionization (MALDI)[5] for analysis. Alternatively, cells on tissue surface were directly analyzed by laser ablation electrospray ionization technique[13,14] and by MALDI/LDI-MS imaging techniques.[15−18] In these studies, however, analyses were performed on cells on detached organs or tissues. Since water status and downstream metabolism are altered after excision of organs/tissues, a means for efficient and less-invasive *in situ* single cell analysis on living plants has yet been demanded, and in this regard, pressure probe electrospray ionization (PPESI)-MS is a promising technique.

As reviewed elsewhere,[19] cell pressure probe was originally designed to directly assess water status and several cellular properties of intact plant cells including turgor pressure, cell wall elasticity, hydraulic conductivity of plasma membrane, and

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cell volume. Hence, this technique allows us to distinguish intactness of targeted cells based on their water status before sampling of cell sap for metabolite analysis. During pressure probe operation, the use of a finely tapered quartz microcapillary mounted on a three-dimensional piezo-manipulator enables highly accurate control of the tip location while penetrating into a cell, and a pressure transducer coupled with variable-speed plunger rod facilitates precise handling of the sample volume inside the microcapillary at the subpicoliter order.[5,6,19] In addition, the volume of cell sap trapped in the microcapillary tip after pressure probe operation can be accurately determined. Gholipour et al.[6] previously reported successful detection and identification of a number of metabolites from parenchyma cells of tulip bulbs using external electrode capillary (EEC)-PPESI-MS system, in which high voltage was supplied to cell sap sample in the pressure probe microcapillary via a metal wire coiled around the tip, to demonstrate reliability of the technique in single cell metabolite profiling. Although relatively homogeneous population of larger cells was studied in the previous work, this technique is potentially applicable to universal types of plant cells showing a variety of morphologies and cellular properties. In this context, plant trichomes are considered as an ideal model cellular system which also has biological and industrial significances.

Trichomes are unicellular or multicellular outgrowths originating from aerial epidermal cells of stems, leaves, floral organs, and fruits of many plant species.[20−22] In *Solanum* spp., they are typically classified into eight distinct types based on their diverse morphological appearance, all of which can be divided into either glandular or nonglandular types depending on the presence or absence of glandular cells atop the stalk cells. Glandular trichomes encompass type I, IV, VI, and VII trichomes, and the rest is regarded as nonglandular types[23] (see Figure S1 in the Supporting Information). Over the past few decades, a growing interest in eco-physiological functions, metabolite biosynthesis, and genetic variations in glandular trichomes has stimulated extensive omics studies on glandular cells, owing to their ability to synthesize and accumulate specialized metabolites of potential importance for pharmaceutical industries and antiherbivory defenses of tomato plants.[22,24,25] To date, a number of specialized compounds including acyl sugars,[18,26−29] terpenoid,[25−27] and flavonoids[27,30] have been documented by metabolomics studies on glandular trichomes. In general, a large quantity of glandular heads prepared by elaborative isolation procedures[25,27] or by gross extraction with purification and separation steps[25−30] was classically required for metabolite analysis, and only recently, metabolite profiling of a single glandular cell has been described using LDI-MS imaging technique.[18] Although these earlier studies compiled knowledge on metabolism in glandular cells, attempts for metabolite analyses with particular emphasis on stalk cells has been completely lacking thus far, and thereby variability of cellular metabolism between the two cell types and among stalk cells of different trichome types are remaining unexplored. This is primarily due to difficulties in selective sampling or isolation of stalk cells without contamination of adjacent glandular or epidermal cells. Toward a more complete understanding of cellular metabolism, functions and differentiation patterns of various trichome types to fully exploit potential benefit of them, it is necessary to evaluate metabolism not only in glandular cells but also in stalk cells.

In this study, the PPESI-MS setup previously described[6] was improved in terms of the detection sensitivity, sample manipulation, and throughput capability to be an eligible tool for single cell metabolomics of intact trichomes. Two major modifications: (i) replacing the external wire electrode capillary (EEC) to an internal electrode capillary (IEC) and (ii) utilizing an ionic liquid to enhance the electric conductivity of silicone oil, were made. By taking advantage of the present IEC-PPESI-MS system, a comparative metabolite profiling in stalk and glandular cells of intact trichomes on tomato plant (*Solanum lycopersicum* L.) was performed to clarify variability in cellular metabolism among different cell types as well as trichome types. To the best of our knowledge, this is the first report describing metabolite profiles of both cell types in tomato trichomes at single cell resolution.

EXPERIMENTAL SECTION

Chemicals and Reagents. All the standard chemicals and organic solvents used in the experiments were LC/MS grade purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ultrapure water of 18.2 MΩ cm^{-1} was used throughout the experiment. For IEC-PPESI-MS operation, an ionic liquid, trihexyl (tetradecyl) phosphonium bis trifluoromethanesulfonyl amide (Cyphos IL109 Strem Chemicals Inc., MA), was suspended in phenyl methyl silicone oil (Wacker silicone fluid AS4, Munich, Germany) at the concentration of 0.01% (v/v) to enhance electric conductivity of the nonpolar silicone oil. For comparison of detection sensitivity between previous and present settings, 10% (v/v) engine oil supplement (MolySpeed 21, Sumico Co., Tokyo, Japan) in silicone oil was prepared according to Gholipour et al.[6]

Plant Materials. The tomato cultivar "Micro-tom" (*S. lycopersicum* L. cv. Microtom) was selected because of its short life span and small plant size at full maturity. The seeds were sown on a humidified growth medium consisting mainly of peat moss (Sakata Seed Corp., Kanagawa, Japan) and incubated in a growth chamber (MLR-352H, Panasonic Corp., Osaka, Japan) where air temperature, relative humidity, photosynthetic photon flux density, and photoperiod were maintained at 28 °C, 60%, 100 μmol m^{-2} s^{-1}, and 16 h, respectively. Two weeks old seedlings were then transplanted to 1 L pots filled with the same growth medium. The pots were supplied with distilled water daily and diluted nutrient solution (HYPONeX, Hyponex Japan Corp., Osaka, Japan) twice a week. In the following experiments, type I trichomes on stems near calyx, type II trichome on adaxial surface of fully matured leaves and type IV and VI trichomes on ripening fruits were subjected to the analysis (Figure S1). The single cell analysis was made on the stalk cells that all types of trichomes possess and on the glandular cells of type I, IV, and VI trichomes. Data were collected from at least three cells on three independent plant replicates.

IEC-PPESI-MS Setup. The basic mechanical setup for PPESI-MS was reported previously.[6] The newly proposed setup and procedures are described as follows and in Figure S2. A quartz microcapillary of 1.0 mm in outer diameter and 0.7 mm in inner diameter was modified with a laser micropipet puller (P-2000, Sutter Instrument Co., CA) to generate a fine tip with an opening diameter of 2−5 μm. The microcapillary was then filled with the 0.01% (v/v) ionic liquid/silicone oil mixture and fitted airtight to a capillary holder in which titanium wire of 0.3 mm in diameter was pre-embedded, so that the internal wire electrode was inserted into the microcapillary through the rear opening and settled near the tip end (Figure 1A,B). Note that the wire electrode was externally coiled around the tip in the

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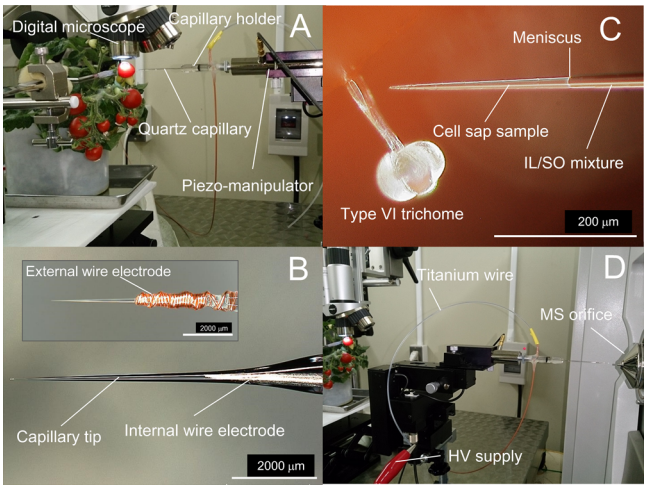

Figure 1. Mechanical setup of pressure probe system (A), quartz capillary tip with internal titanium wire electrode used in the current study (B), capillary tip containing sampled sap from type VI stalk cell (C), and subsequent ionization/detection by Orbitrap MS (D). The inset photograph in part B shows the tip with external copper wire electrode capillary reported in Gholipour et al.[6]

previous setup (Figure 1B, inset). Upon the measurement, a potted plant was placed on a three-axis translation sample stage, and the stems, leaves, or fruits were clamped with tweezers, if necessary, to minimize vibration under a digital microscope (KH-8700, HiRox Co. Ltd., Tokyo, Japan). The tip was then impaled into targeted cells with the aid of a motorized piezo-manipulator (DC-3K, Märzhäuser Wetzlar, Germany). When the meniscus between the cell sap and the ionic liquid/silicone oil mixture was visually observed, the cell turgor pressure (Ψ_p) was measured as described previously.[19] Subsequently, the microcapillary was depressurized to collect the cell sap, and the tip containing sample was pulled out of the cell (Figure 1C). The microscopic images of the tip was captured for sample volume calculation based on tip geometry and the position of meniscus as described previously,[6] and then the tip was carefully oriented toward the orifice of an orbitrap mass spectrometer (Exactive Plus, Thermo Fisher Scientific Inc., MA) at approximately 3 mm distance (Figure 1D). The tip was then electrified with −4 kV via the internal wire electrode connected to a high voltage generator (AKTB-05k1PN/S, Touwa Keisoku Corp., Tokyo, Japan). The MS scan was performed in negative ion mode with the instrumental settings of 200 ms as maximum injection time, inlet ion transfer tube temperature of 250 °C, and resolution of 35 000. When the targeted cell was punctured successfully without tip plugging, the entire process of IEC-PPESI-MS analysis on a stalk cell can be completed within a few minutes as there is no need of extra sample preparation and/or extensive rearrangements of mechanical setup for ionization.

Metabolite Profiling of Glandular Cells of Types I, IV, and VI Trichomes. In type I and IV trichomes, extremely viscous and sticky glandular secretion is deposited on the extracellular surfaces of glandular heads. Because of its high viscosity, direct sampling of the secreted material into the microcapillary and subsequent electrospray ionization were unfeasible. For this reason, the glandular secretion of types I and IV was sampled on the external wall of capillary tip with gentle contact (Movie S-1). The tip was then briefly dipped into a 500 nL droplet of ultrapure water formed at a micropipet tip, and at the same time, approximately 300 pL of the solution was loaded into the microcapillary (Movie S-2). By using this sampling method, secreted material was instantly and locally dissolved into the water droplet, and the diluted sample was simultaneously trapped into the pressure probe microcapillary. For glandular cells of type VI trichome which often rupture and stiffen immediately after tip penetration, a portion of stiffened cellular component was sampled on the tip surface and then briefly extracted in ultrapure water as described above. After the sampling, negative ion mode measurements were performed.

Identification of Metabolites. The list of monoisotopic exact m/z values for all the peaks on acquired mass spectra were extracted using "Qual Browser" application in the Thermo Xcalibur software (Thermo Fisher Scientific Inc., MA). The observed peaks were then matched with theoretical masses of candidate metabolites in the online metabolomics databases, namely, Metlin (http://metlin.scripps.edu/index.php), Plant Metabolite Network (http://pmn.plantcyc.org), and Solcyc (http://solcyc.solgenomics.net/), as well as lists of metabolites in tomato trichomes previously reported.[18,26−29] The putative metabolites were also confirmed with simulated isotopic ratios using the"Qual Browser" application. Additionally, the collision-induced dissociation (CID) tandem MS analysis on putative acyl sugars in glandular secretion of type IV trichomes and flavonoids in stalk cells of type VI trichomes were performed with an Orbitrap Velos Pro (Thermo Fisher Scientific Inc., MA) coupled with IEC-PPESI for further identification. To ensure adequate duration of electrospray ionization for selection and fragmentation of precursor peaks, crude cell sap was diluted in ultrapure water as described above. The acquired MS/MS fragmentation patterns were consulted with the online database and literature.[28,31−33]

Determination of Detection Sensitivity of the System. For determination of detection sensitivity for newly developed IEC-PPESI setup, solutions with different concentrations of glucose, sucrose, citric acid, malic acid, nicotinic acid, and hydroxyl-proline were prepared. Then, 10 pL of each standard solution was loaded into the microcapillary with the aid of a micropipet and injected to the MS with the same settings as described above. If peaks of each analyte appeared on the mass spectra, the analyte was considered as detectable at a given concentration, and solution with lower concentration was tested. The dilutions were successively made, and the measurement was conducted until no analyte signal appeared in the spectrum. The change in detection sensitivity by adoption of modified capillary holder was also evaluated with 100 pL of sucrose and malic acid standard solutions in positive and negative ion modes, respectively (Figure S3). In this case, the areas of chromatogram of selected peaks for each standard were taken into account. In addition, detectable lowest concentrations of sucrose and malic acid were also determined using internal electrode capillary filled with the engine oil mixture and the ionic liquid mixture to examine the effect of different supporting electrolyte on the system sensitivity (Table S1).

Statistical Analysis. All quantitative data including cellular dimensions, sampled sap volume and turgor pressure was subjected to Tukey's range test using Sigma Plot 12 (Systat Software Inc., CA).

RESULTS AND DISCUSSION

IEC-PPESI-MS System Development. The adoption of the ionic liquid mixture was found more suitable than the

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engine oil mixture in the following aspects. First, the commercially available engine oil supplement contains many unknown ingredients which result in complex background peaks with high signal intensities. The IEC-PPESI negative ion mass spectrum of ultrapure water obtained using a capillary filled with the engine oil mixture showed background peaks at m/z 241, 297, 353 and cluster with 14 Da intervals as well as many minor peaks at the lower m/z range (Figure S4A). In contrast, a single dominant peak at m/z 279 from bis-(trifluoromethanesulfonyl) amide, the anionic counterpart of the ionic liquid, was detected in the negative ion mass spectrum obtained using a capillary filled with the 0.01% ionic liquid mixture (Figure S4B). Reducing background peaks is ideal for detection and identification of unknown metabolites in complex biological samples especially in a nontargeted shotgun metabolomics devoid of purification steps. Both blank mass spectra obtained with engine oil and ionic liquid mixtures in positive ion mode showed higher background peaks (Figure S4C,D) than in negative ion mode. Therefore, IEC-PPESI-MS measurements on tomato trichomes were preferentially performed in negative ion mode.

Lipophilic ionic liquids were previously utilized as a supporting electrolyte to enhance electric conductivity of nonpolar organic solvents such as benzene, hexane, and toluene for ESI-MS.[34,35] Recently, unique electrochemical properties of ionic liquids were also applied to solvent-free probe electrospray ambient ionization-MS in the field of green chemistry.[36] In the present study, trihexyltetradecyl phosphonium bis-(trifluoromethylsulfonyl) amide, 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide, and 1-ethyl-3-methyllimidazolium dicyanamide, which differ in miscibility in the silicone oil, were also compared for their performance in IEC-PPESI-MS on the basis of signal intensity and stability (Table S2). Among those tested, trihexyltetradecyl phosphonium bis-(trifluoromethylsulfonyl) amide yielded the highest signal intensity (Figure S5). Moreover, the minimal concentration of this ionic liquid to obtain measurable signal intensity of standard metabolites was less than 0.0025% (Figure S6). As a result of utilization of the ionic liquid mixture, sensitivity of the IEC-PPESI-MS was improved approximately 10 times as compared to engine oil mixture (Table S1).

Besides the utilization of the ionic liquid as a supporting electrolyte in silicone oil, the modification of the capillary holder allowed direct installation of pressure probe capillaries without extra preparation (i.e., coiling metal wire around the tip) by which replacement of plugged, damaged, and used capillaries was drastically simplified, improving throughput performance of the technique. This improvement was particularly important when cells possessing viscous cell sap and harder cell wall, such as glandular head of type VI trichome and stalk cells of type IV trichome, were targeted for analysis. In these cases, frequent tip plugging followed by tip breakage and replacement were commonly observed.

As compared to the previous electrode-capillary setup, the newly developed capillary holder ensures direct electrification of the sample solution through the ionic liquid mixture inside the capillary. Consequently, the capillary holder modification led to a 30- and 52-fold increase in detection sensitivity for positive and negative ion modes, respectively (Figure S3). As a combined effect of utilization of the new capillary holder and the ionic liquid mixture as well as installation of the upgraded MS with higher sensitivity, the overall detection sensitivity of the system was substantially enhanced. The detectable amount of some standard metabolites were as low as attomoles order (Table 1), whereas the values reported in the previous system settings were femto- to picomoles order.[6]

Table 1. Detectable Lowest Amount of Standard Metabolites Obtained with the Previously[6] Reported Setup (EEC-PPESI with the Engine Oil Mixture) and Current Setup (IEC-PPESI with the Ionic Liquid Mixture)[a]

	positive ion mode		negative ion mode	
standard	current	from ref 6	current	from ref 6
ascorbic acid	1	32 000	0.01	40
citric acid	0.5	3 000	0.005	30
glucose	0.1	500	0.01	110
hydroxyproline	0.025	3 000	0.005	40
malic acid	1	162 000	0.0025	500
nicotinic acid	0.05	3 000	0.001	30
sucrose	0.01	1 200	0.005	500

[a]All values are in femtomoles.

Sequential Metabolite Profiling of Different Cell Types in an Intact Type VI Trichome. IEC-PPESI-MS analysis was performed individually on stalk and glandular cells of an intact type VI trichome to demonstrate the validity and less-invasiveness of this technique for *in situ* single cell metabolomics of living trichomes. The measurement was first made on the stalk cell and then on the adjacent glandular cell of the same trichome. The spatial distance between two cell types was 22.97 μm on average (n = 7). To avoid severe disturbance on trichome structure, the minimal sample volume ranging from sub-picoliter to a few picoliter was obtained from targeted stalk cells. As compared to easily accessible and relatively large glandular cells of type VI trichomes, obtaining sap from the stalk cell required a more precise three-dimensional control of tip location that would be almost impossible to achieve without the aid of the high resolution digital microscope and remotely controlled piezo-manipulator settled on a magnetically floated vibration-free table (Figure 1A). With this precise sampling method, single cell sap from the intact stalk was obtained and ionized without contamination of the neighboring glandular cell.

Several hundreds of peaks were detected in a negative ion mode IEC-PPESI mass spectrum with sub-picoliter sap of stalk cell (Figure 2A), in which five classes of metabolites including amino acids (i.e., asparagine m/z 131, glutamine m/z 145, etc.), organic acids (i.e., malic acid m/z 133, citric acid m/z 191, etc.), carbohydrates (i.e., Hex m/z 179, Hex_2 m/z 341, etc.), phenolic and flavonoid precursors (i.e., shikimic acid m/z 173, quinic acid m/z 191, etc.), and flavonoids (i.e., naringenin-hexoside m/z 433, quercetin-hexosyl-hexoside m/z 609, etc.) were identified at less than 5 ppm differences from theoretical values (Table S3 and Figure S6). As compared to the stalk cell, the corresponding glandular cell on the same trichome showed a strikingly different mass spectrum in which prominent peaks of doubly- and singly deprotonated quercetin-hexosyl-hexoside (m/z 304 and m/z 609) were detected concomitantly with relatively lower peaks of organic acids (i.e., malate m/z 133, citrate m/z 191, etc.) (Figure 2B and Table S3). These results clearly depicted the presence of significant differences in metabolites abundance between the neighboring stalk and glandular cells within the same trichome. It is also noteworthy that only sub-picoliter cell sap was sufficient for metabolite

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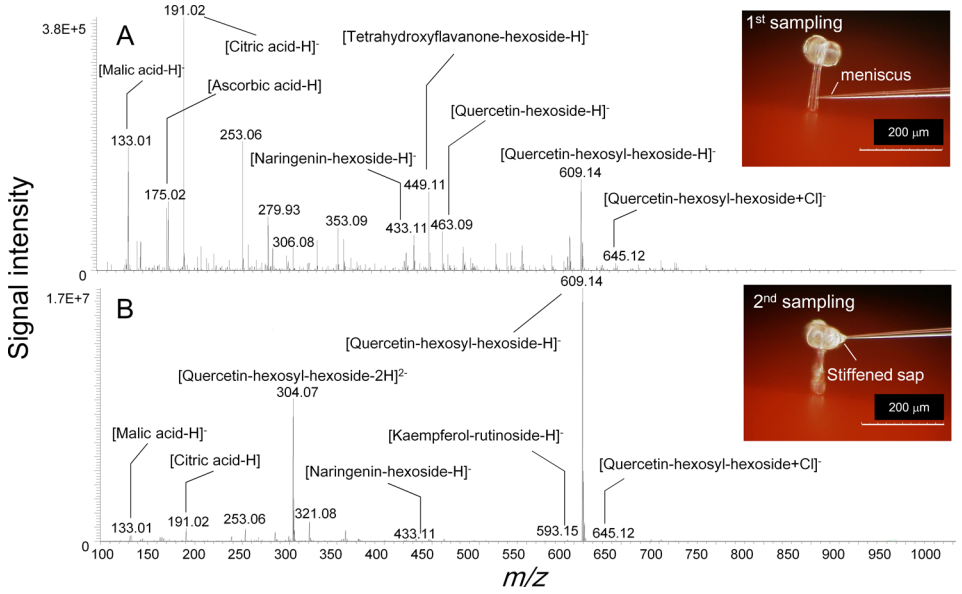

Figure 2. IEC-PPESI negative ion mode mass spectra successively obtained from stalk (A) and glandular cells (B) of an intact type VI trichome. Sampling was carried out first from the stalk cell and then the glandular cell as indicated by the inset photographs.

Table 2. Upper and Lower Diameters (Φ_u and Φ_L), Height (h), Cell Volume (V), Sampled Sap Volume (SSV), and Turgor Pressure (Ψ_p) of Stalk Cells from Different Types of Trichomes[a]

type	Φ_u (μm)	Φ_L (μm)	h (μm)	V (pL)	SSV (pL)	Ψ_p (MPa)
I	72.1 ± 2.5[a]	85.5 ± 6.9[a]	931.1 ± 47.9[a]	4729.5 ± 485.7[a]	442.5 ± 82.6[a]	0.58 ± 0.01[a]
II	70.7 ± 2.6[a]	79.6 ± 2.4[a]	454.9 ± 29.7[b]	2155.6 ± 252.3[b]	21.1 ± 3.3[b]	0.40 ± 0.01[b]
IV	21.3 ± 0.5[b]	23.6 ± 0.7[b]	99.0 ± 6.3[c]	40.3 ± 3.5[c]	1.2 ± 0.2[b]	0.10 ± 0.00[d]
VI	19.3 ± 0.5[b]	23.7 ± 0.6[b]	74.0 ± 2.2[c]	27.4 ± 1.7[c]	0.5 ± 0.1[b]	0.15 ± 0.01[c]

[a]Values followed by different superscript letters (a, b, c) are significantly different among trichome types at $P < 0.01$ by Tukey's range test. The values are means ± SE (n = 12−19).

profiling of the stalk cell. This could be largely attributed to significant increase in detection sensitivity of the system. The removal of such minimal quantity of cell sap accounting for approximately 2% of total cell volume would not substantially affect the structure, physicochemical properties, and function of the cell, making this technique as less-invasive. Shackel et al.[37] previously demonstrated that puncture wounds caused by insertion of capillary tip in leaf epidermal cells of *Tradescantia virginiana* L. were rapidly resealed within a few seconds following the tip withdrawal, and Ψ_p of the targeted cell was restored to more than 90% of the initial value in the second Ψ_p measurement. Moreover, pressure probe-aided microinjection of fluorescent probe into living stalk cells of tobacco trichomes succeeded in *in situ* observation of symplasmic cell-to-cell transport.[38] With regard to these reports, our system may be capable of repetitive metabolite profiling in an identical living cell to monitor temporal fluctuation in cellular metabolism.

Comparative Metabolite Profiling of Stalk Cells in Different Trichome Types. Intact stalk cells of different trichome types varied significantly in their location, morphology, cell size, density, and fragility. Therefore, the difficulties of sampling and volumes of collected cell sap also differed significantly among trichome types. All stalk cells targeted for IEC-PPESI-MS analysis showed positive Ψ_p, indicating that these cells were fully intact. The highest Ψ_p was observed in type I, followed by types II, VI, and IV trichomes (Table 2). The Ψ_p of type I trichome was close to previously reported Ψ_p (0.57 ± 0.01 MPa) for type I-like trichomes on tobacco leaves.[38] The higher Ψ_p in type I and II trichomes would be crucial for mechanically maintaining lengthened trichome structure to function as a physical deterrent against herbivorous insects, as structural distortion of trichomes resulted in increased susceptibility to herbivore attack.[39] The single cell sampling was accomplished most straightforwardly in type I stalk cells owing to its large cell size with high turgidity, providing sample volume of several hundreds of picoliters (Table 2 and Figure S1). On the other hand, because of narrow cylindrical shape and the less turgid cell of type IV stalk cells, tip handling for penetration was most technically demanding, and acquisition of cell sap seemed virtually unfeasible without the precise control of oil pressure inside the capillary. The sample from types IV and VI stalk cells generally exhibited higher viscosity than those of type I and II trichomes, causing tip plugging more frequently during the process of sampling and ionization.

The number of detected metabolites and their relative peak intensities varied across trichome types. As claimed in the literature,[27,40] quantifying absolute abundance of each metabolite is impractical due to differences in ionization efficiency and potential ion suppression caused by variable matrix effects. Hence, cell-to-cell variations in metabolite compositions were interpreted semiquantitatively on the basis of detection frequency and relative peak intensity. In type I trichomes, the peak at m/z 133 corresponding to malic acid was detected

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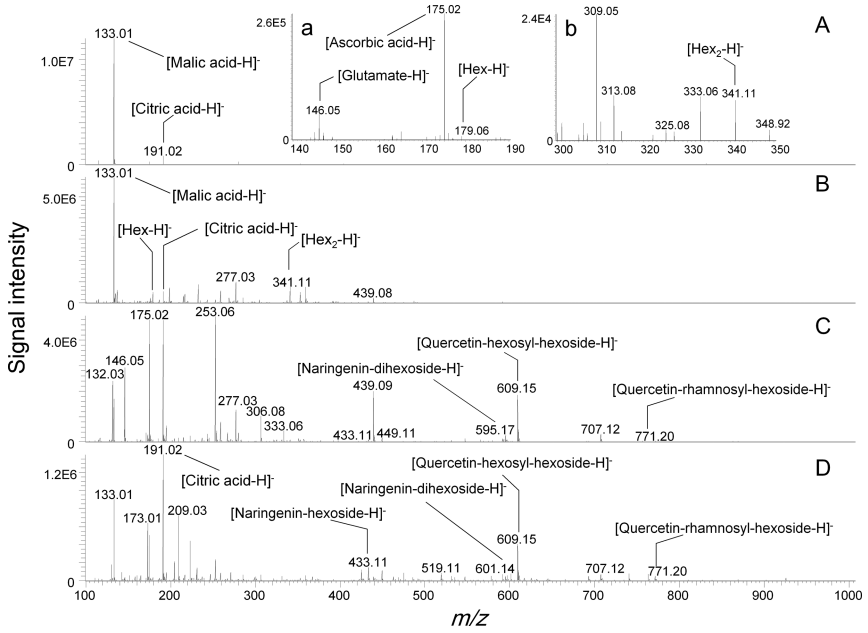

Figure 3. Representative IEC-PPESI negative ion mode mass spectra obtained from a stalk cell of type I trichome on the stem (A), type II trichome on the leaf (B), type IV (C), and VI trichomes (D) on the fruit. The inset figures in part A indicate magnified ranges of m/z 140−190 (a) and 300−350 (b) to show peaks of $[Hex - H]^-$ and $[Hex_2 - H]^-$. The magnified peaks of all the metabolites listed in Table S3 are shown in Figure S6 (Supporting Information).

almost exclusively with a lower peak of citric acid at m/z 191 (Figure 3A, Table S3, and Figure S7). The minor peaks of amino acids, carbohydrates, and other organic acids also appeared intermittently on chromatograms. Regarding the highest Ψ_p of type I trichomes (Table 2), high abundance of malate may be responsible, at least in part, for sustaining turgidity of type I stalk cells, since vacuolar accumulation of malate in stomatal guard cell, an another specialized cell type on epidermal cell in plant leaves and stems, has been documented as a molecular osmoregulator to induce stomatal opening by elevating guard cell turgor.[41] Similar mechanism may be involved in maintenance of high Ψ_p in type I trichomes.

Type II trichomes which morphologically resembles type I trichomes except for the absence of the glandular head (Table 2 and Figure S1) exhibited a similar set of metabolites to type I trichomes (Figure 3B and Table S3). As in type I stalk cells, signal intensity of malic acid was considerably high; however, type II stalk cells tended to show higher relative intensity of hexose and dihexose peaks at m/z 179 and m/z 341, respectively. Since type II trichomes on adaxial leaf surface are apparently devoid of chloroplasts, photosynthates allocated from underlying mesophyll cells were more likely to be the source of these carbohydrates. Other than these minor differences in the mass spectra, no remarkable difference in metabolite compositions between type I and II trichomes were observed (Table S3). This is despite the fact that types I and II trichomes belong to glandular and nonglandular types, respectively, and were derived from different organs (i.e., stems and leaves). The nano ESI orbitrap mass spectra of type I and II stalk cells also showed the similar trends, confirming the reliability of the IEC-PPESI-MS analysis (Figure S8).

Unlike types I and II stalk cells, metabolite compositions of types IV and VI stalk cells were abundant in flavonoids (Figure 3C,D, Table S3, and Figure S10). The intensive accumulation of flavonoids in glandular cells of type VI trichomes, particularly kaempferol-*O*-rutinoside (m/z 593), quercetin-*O*-rutinoside (m/z 609), and quercetin-*O*-diglucosides (m/z 625), has been reported previously;[18,26,27] however, there has been no report describing the presence of flavonoids in stalk cells of any trichome types. On the basis of our findings, 13 flavonoids were detected in single stalk cells of types IV and VI trichomes, and these flavonoids were putatively identified by MS/MS fragmentation patterns and accurate masses (Table S3). Along with those flavonoids, peaks of several precursor metabolites related to flavonoid biosynthesis via phenylpropanoid pathway,[25,30] including shikimic acid (m/z 173), phenylalanine (m/z 164), quinic acid (m/z 191), chlorogenic acid (m/z 353), and naringenin/naringenin chalcone (m/z 271) were detected (Table S3), suggesting a possibility of active flavonoid biosynthesis in stalk cells of both type IV and VI trichomes. The combined analyses of transcriptome and metabolome in isolated glandular cells from type VI trichome previously suggested that the *de novo* synthesis of flavonoids in secretory cell may be independent of sucrose supply from stalk cells in *S. lycopersicum*.[27] In the light of our results, the intercellular translocation of flavonoid glycosides synthesized in stalk to glandular cells, and vice versa, may be a potential and alternative mechanism of flavonoids accumulation in type VI trichomes. Although pathways and regulatory mechanisms of intercellular flavonoid trafficking are still poorly understood, the key involvement of glutathione *S*-transferase which mediates formation of flavonoid-glutathione conjugates was pointed out recently.[42] Regarding this report, the higher detection frequency of glutathione in type VI trichomes than in type I trichomes partially supports our hypothesis (Table S3).

In stalk cells of types IV and VI trichomes, the detection frequencies of galacturonic acids, the polymerized form of which is known as pectin, were also higher than those in type I and II trichomes. This observation may explain high viscosity of the cell sap in types IV and VI trichomes which caused frequent tip plugging. Further comparison of metabolite profiles between types IV and VI stalk cells revealed that high detection

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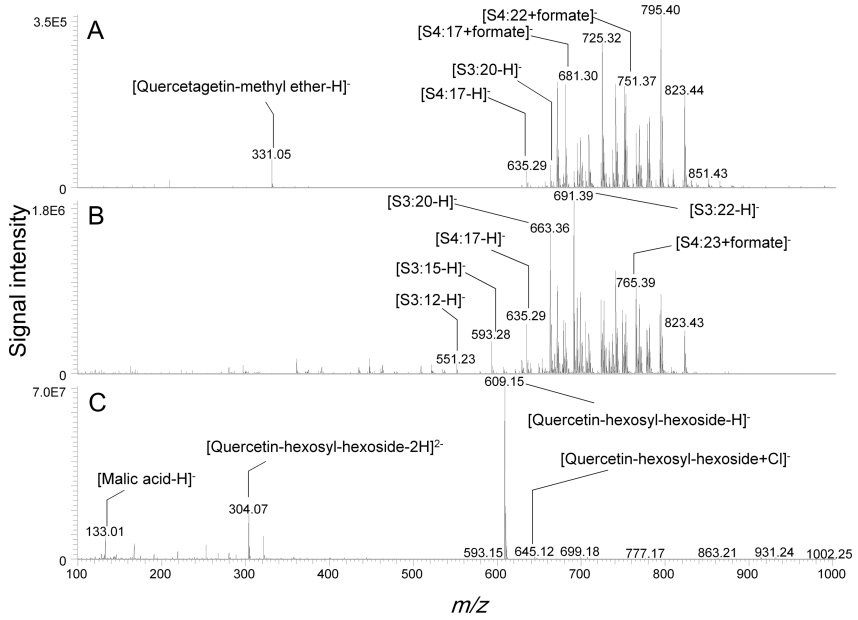

Figure 4. Representative IEC-PPESI negative ion mode mass spectra obtained from glandular cells of type I trichome on the stem (A) and type IV (B) and VI trichomes (C) on the fruit. The magnified peaks of acyl sugars listed in Table S3 are shown in Figure S8 (Supporting Information).

frequencies of deprotonated Hex_3 (m/z 503), Hex_4 (m/z 665), and Hex_5 (m/z 827) were unique to type VI trichomes (Figure 3D, Table S3, and Figure S6). Interestingly, many of carbohydrates and flavonoid glycosides were frequently detected as $[M + Cl]^-$ in stalk cells of type VI as well as type IV trichomes. Chlorinated ion formation of glycolipids[43] and carbohydrates[44,45] in the presence of Cl^- has been formerly documented with ESI- and MALDI-MS. In addition, Cl^- adduct ions of carbohydrate, flavonoid glycosides, alkaloids, and acyl sugars were also reported in the trichome extract prepared by dipping leaflets in organic solvent.[27] Since sequestration of chloride ions was reported as an important physiological role of specialized trichomes called bladder cells in some halophyte,[46,47] a similar function of tomato trichomes can be suggested from these results.

Metabolite Composition in Glandular Cells of Types I, IV, and VI Trichomes. In agreement with the literature reporting metabolite profile in glandular cells of different types of tomato trichomes using HPLC/MS and ^{1}HNMR with collectively isolated glandular head[27,29] and single-cell LDI-MS imaging,[18] type I and IV glandular cells were rich in acyl sugars (Figure 4A,B, Table S3, and Figures S8 and S10). In type I trichome, 14 acyl sugars were detected with high intensity, and a similar set of acyl sugars was found in type IV trichome. In contrast, no acyl sugars were detected in type VI trichome (Figure 4C and Table S3). Such selective accumulation of acyl sugars in type I and IV glandular cells, but not in type VI glandular cell, is consistent with a previous report.[18] Although glandular secretion collected on the tip of microcapillary was subsequently diluted with pure water for ionization in this study, the concept of solvent-free probe electrospray ionization technique with aid of ionic liquids[36] may be an alternative approach for direct ionization and detection of sticky cell samples.

Apart from acyl sugars, composition of flavonoids in those glandular cells also showed variation among trichome types. While type VI glandular cells accumulated a complete set of flavonoids detected in stalk cells, glandular cell of the type IV trichome showed only two flavonoids observed in stalk cells and two flavonoids which were not observed in stalk cells, namely, quercetin aglycone and quercetagetin-methyl ether (Table S3); the latter compound was also the only flavonoid found in type I glandular cells. Because an antiviral property against tomato bushy stunt virus has been reported for quercetagetin-methyl ether in *Nicotina* species,[48] accumulation and secretion of this phytochemical from glandular heads would be physiologically important. Here, it should be reemphasized that quercetagetin-methyl ether as well as acyl sugars were not detected in corresponding stalk cells of any trichome types, and therefore, those metabolites were locally synthesized, accumulated, and secreted by glandular cells (Table S3). The above results provided compelling evidence for remarkable differentiation in cellular metabolism between two cell types within a unit of trichome. However, it should be noted that, for glandular cells of type I and IV trichomes, secretion deposited on the extracellular surface was sampled, whereas intracellular components were analyzed for stalk cells. Thus, metabolic differences between two cell types were possibly influenced by the presence of secretion. Even with the aid of pressure probe sampling technique, it is still difficult to obtain pure cell sap of types I and IV glandular cells due to sticky secretion covering small glandular cells.

■ CONCLUSIONS

In this contribution, we demonstrated the metabolite profiling of adjacent stalk and glandular cells in intact trichomes that had never been attempted due to technical limitations. The successful single-cell analysis was largely owing to the outstanding properties of the pressure probe for intact single cell sampling and the utilization of the new electrode capillary for ionization which enabled sensitive detection of cellular metabolites in high-throughput manner. Our findings clearly indicated that trichomes are differentiated not only in their morphologies but also in their turgidity, physicochemical properties of cell sap, and metabolite compositions. Because significant variations in metabolite profiles of glandular

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trichomes across *Solanum* species and genotypes have been reported previously,[27] our curiosity is further extended to how these cell-to-cell variations are influenced by genotypes as well as by the fluctuating environment. To this end, further technical improvements for quantitative IEC-PPESI-MS analysis with installation of either purification steps or internal standard method are critical. Future application of this technique may accelerate understanding of cellular metabolism in trichome as well as other specialized cells in plants and animals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03366.

Supplementary tables and figures (PDF)
Movie S1: Sampling of type IV glandular cell (ZIP)
Movie S2: In capillary dilution of the sample (ZIP)

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Author Contributions

The experiments were designed by H.N., R.E.-B., and T.N. and were performed by T.N. The manuscript was written and edited by all the authors.

Notes

The authors declare no competing financial interest.

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