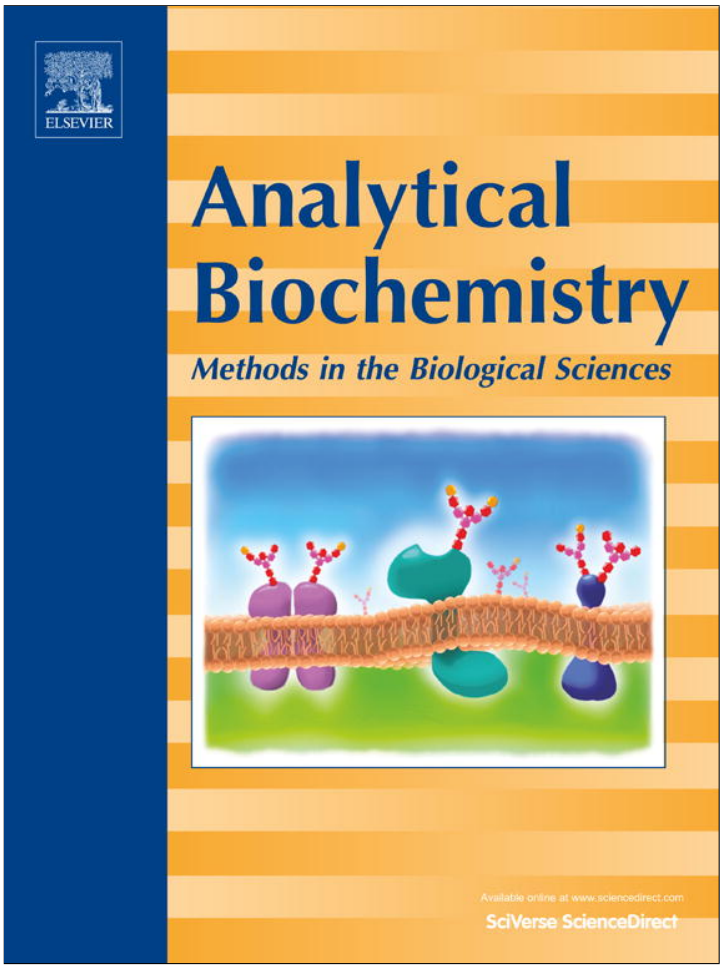

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Living cell manipulation, manageable sampling, and shotgun picoliter electrospray mass spectrometry for profiling metabolites

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ABSTRACT

A modified cell pressure probe and an online Orbitrap mass spectrometer were used to sample in situ plant single cells without any additional manipulation. The cell pressure probe, a quartz capillary tip filled with an oil mixture, was penetrated to various depths into parenchyma cells of tulip bulb scale, followed by a hydraulic continuity test to determine the exact location of the tip inside target cells. The operation was conducted under a digital microscope, and the capillary tip was photographed to calculate the volume of the cell sap sucked. The cell sap sample was then directly nebulized/ionized under high-voltage conditions at the entrance of the mass spectrometer. Several sugars, amino acids, organic acids, vitamins, fatty acids, and secondary metabolites were detected. Because picoliter solutions can be accurately handled and measured, known volumes of standard solutions can be added to cell sap samples inside the capillary tip to be used as references for metabolite characterization and relative quantitation. The high precision and sensitivity of the cell pressure probe and Orbitrap mass spectrometer allow for the manipulation and analysis of both femtoliter cell sap samples and standard solutions.

Analysis of the physical and chemical properties of a cell with single-cell resolution can be used to distinguish cell-to-cell variations and many primary growth, disorder, or stress-related phenomena that cannot be detected or fully explored through tissue-level studies. Although genomic and proteomic analyses of single cells have been established, developing analytical techniques for single-cell metabolomics is still the subject of active research [1]. Nuclear magnetic resonance (NMR)[1] and mass spectrometry (MS) techniques have classically been applied for metabolome analysis at the tissue level. Due to the high sensitivity, resolution, and dynamic range of detection, MS is the top choice. Combined chromatography–MS techniques that employ liquid chromatography (LC) or gas chromatography (GC) for separating, characterizing, and quantifying biomolecules are well established (LC–MS and GC–MS). However, the so-called shotgun approach for mixture analysis has attracted the attention of omics scientists due to its minimized preparations and speed. Although the availability of mass spectrometers with high sensitivity is a must for the detection of metabolites in cell sap samples, reliable single-cell sampling is also a major challenge. Because the volume of a cell sap sample varies in the range of femtoliters to nanoliters, accurate localization of the sampling capillary and minimal loss of the sample due to handling should be taken into account. In addition, the volume of the sample must be measured if the analyses are aimed at clarifying the natural changes in the abundance of metabolites. Furthermore, the sampling of plant cells faces additional difficulties owing to the properties of the cell walls.

Reports describing successful in situ plant tissue and cell sampling followed by shotgun metabolite profiling are available. Probe electrospray MS (PESI MS) was able to pick up in situ tissue samples by penetrating samples with a micrometer-order metal needle [2]. Although the sampling and metabolite profiling of plant tissues was successfully carried out with PESI MS [3], the sample volume could not be measured when using this technique. Penetration of a cell by a nanoESI capillary tip, followed by the direct electrospraying of cell sap samples with the aid of a solvent, has been established [4,5] and applied to plant cells [6]. The advantage of the latter technique is that the localization of the tip into the target cell was conducted under a microscope. Although the volume of sample was not measured, researchers could elucidate the relative abundance of the examined metabolites. In laser ablation electrospray ionization (LAESI), mid-infrared laser pulses were delivered through the tip of a glass fiber after penetrating the tip

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[1] *Abbreviations used:* NMR, nuclear magnetic resonance; MS, mass spectrometry; LC, liquid chromatography; GC, gas chromatography; PESI MS, probe electrospray MS; UV, ultraviolet; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization; Si oil, silicon oil; LOD, limit of detection.

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into the cells [7]. The operation was monitored by a camera, making it possible to select the target cell and to accurately localize the tip. In this technique, the sample volume could not be measured, and single-cell sampling with high accuracy could be performed only on epidermal cells.

The calculation of the volume of cells by using a cell pressure probe was described previously by Nonami and Boyer [8]. A similar calculation for picoliter samples gathered with a capillary from plant cells for MS-based metabolite profiling was recently described [9,10]. We have used a pressure probe for single-cell sampling followed by ultraviolet matrix-assisted laser desorption/ionization time-of-flight (UV–MALDI–TOF) MS analysis for metabolite profiling [11–13]. The pressure probe is routinely used to analyze several properties of plant single cells, including turgor [14–16], osmotic potential [17,18], water potential [18], cell wall elastic modulus [15,16,19], and cell wall extensibility [14,15,20], hydraulic conductivity of plasma membrane [14–16,21], and in situ cell volume [8,21]. In addition, pressure probe facilitates picoliter sampling of in situ single cell because the sample volume can be controlled and measured [18]. The advantage of the application of cell pressure probe combined with UV–MALDI MS was the fully manageable femtoliter to picoliter sampling and the ability to access deep cells. However, the analysis was offline and required several sample preparation steps, including the transfer of picoliter cell sap samples onto the MALDI plate and the selection of the proper MALDI matrix. Because MALDI matrices are not necessarily universal, only a fraction of the total metabolites were detected per cell sample with each specific matrix.

The electrification of liquid at the end of a capillary tube was described by Zeleny in 1917 [22] and was followed several years later by the development of electrospray ionization (ESI) MS [23,24]. By pulling a glass capillary, Wilm and Mann made tapered capillaries with tips of only a few micrometers in diameter and developed a new ion source for electrospraying analyte solutions with flow rates of approximately 25 nl/min [25]. The technique was later called nanoESI [26]. We decided to apply a high voltage directly to the tip of the quartz capillary of the pressure probe and its contents to conduct metabolite profiling with an Orbitrap mass spectrometer. We selected the tulip as the model plant because there were previous reports on the detection of metabolites in the bulb tissue samples with PESI MS [3] and the cell sap samples with UV–MALDI–TOF MS [11] with which the results of this work could be compared. Furthermore, complementary pressure probe sampling and nanoESI Orbitrap MS metabolite profiling of single-cell sap from tulip bulbs was also conducted for comparison of both techniques and for further confirmation and peak assignment.

Materials and methods

Plant material

Tulip bulbs (*Tulipa gesneriana* L. cv. Oxford) were purchased in August. The bulbs were split into two groups, with 600 bulbs precooled at 15 ± 0.5 °C for 2 weeks and 600 bulbs stored as the control in a ventilated dark chamber whose temperature was kept steady at 25 ± 0.5 °C. The precooled bulbs were then stored in a

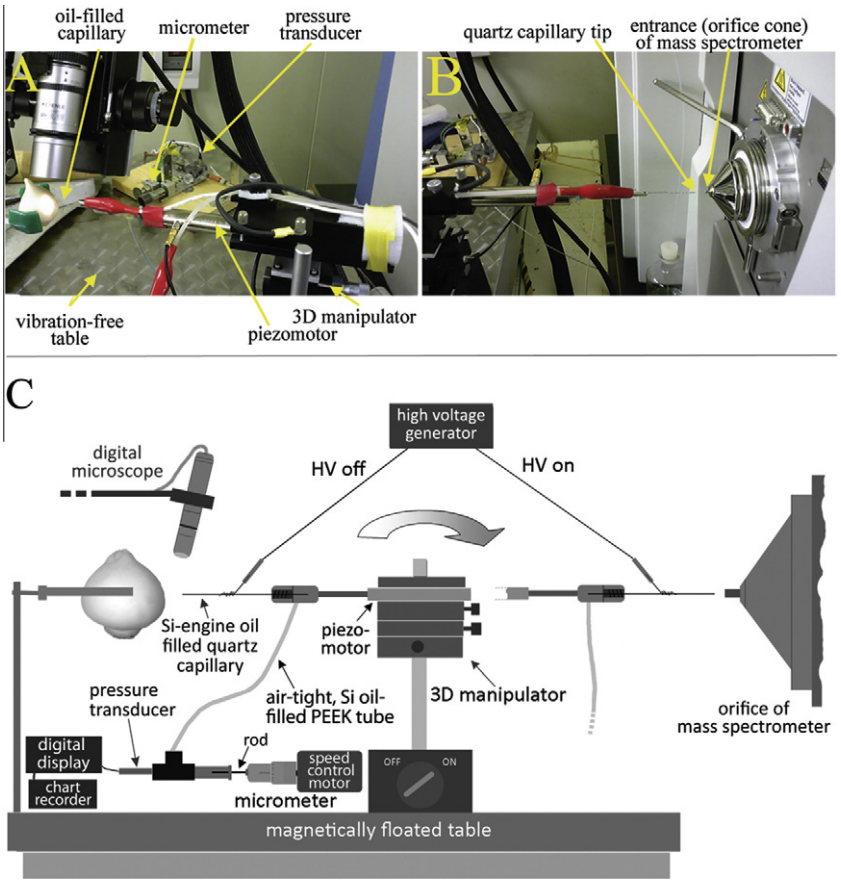

Fig.1. The cell pressure probe and the plant stand are placed on a vibration-free table. The quartz capillary is filled with the mixture of Si oil and engine oil supplement (9:1, v/v), whose volume is changed by a rod moving back and forth with a motorized micrometer. The pressure transducer and the capillary are connected with a flexible, air-tight, Si oil-filled PEEK (polyetheretherketone) tube. After single-cell sampling (A), the manipulator is flipped around horizontally toward the mass spectrometer entrance (cone orifice), and high voltage (HV) is applied on the pressure probe quartz capillary to nebulize/ionize the picoliter sample at the atmospheric pressure (B). A general scheme of the instrument is shown (C).

refrigerator at 5–7 °C for 9 weeks. The bulbs from the 8th week of the cold storage were used for single-cell analysis.

Sampling

Fig. 1 shows the setup of pressure probe and picoliter ESI MS. Details of sample management and pressure probe operation can be found in the Supplementary material and in our previous article [12]. In the case of tulip bulb cells, a quartz capillary (Sutter Instrument, Novato, CA, USA) with a 1.0-mm outer diameter, a 0.7-mm inner diameter, and a 3- to 7-μm tip outer diameter was selected. The diameter of the target cells was approximately 50–150 μm (Fig. 2); therefore, the selected tip size was suitable for single-cell sampling of our model plant. Subepidermal, sugar-storing parenchyma cells at a depth of approximately 400 μm from the cuticle were sampled from the second scale layer from the outer surface of the tulip bulbs (Fig. 2). The tip is penetrated into parenchyma cells, and a meniscus is created at the interface of the cell solution and the oil mixture (Fig. 3B). Before collecting and extracting the cell samples, the true penetration of the capillary into the cell and the absence of leakage were confirmed by a hydraulic continuity test (see details in Supplementary material; Movie S-1). The pressure probe capillary tip with picoliter cell sap sample or standard solution is photographed before applying high voltage, and cell sap volume is accurately measured (Fig. 4; see calculation in Supplementary material).

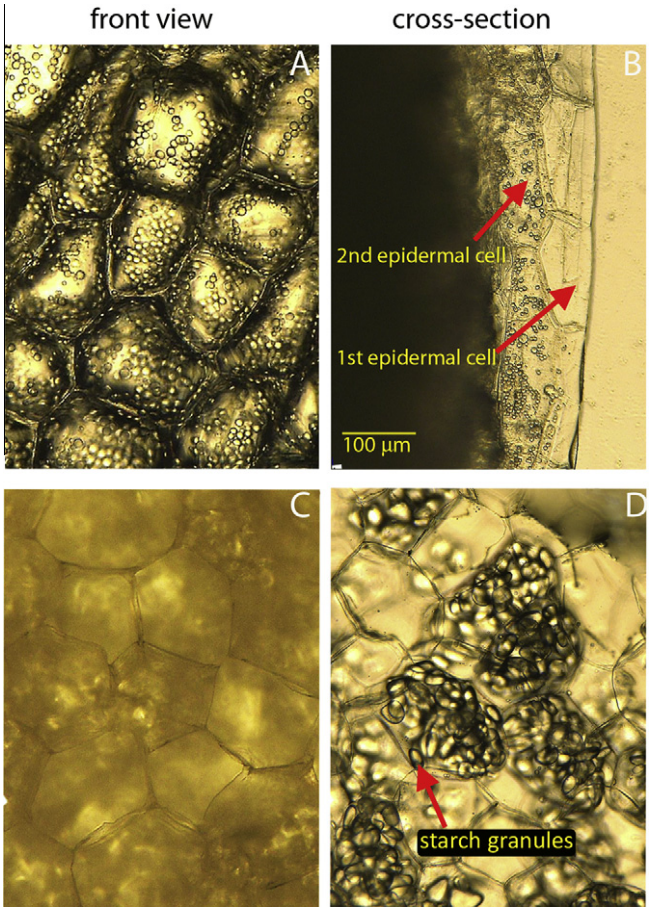

Fig.2. Anatomy of the second scale of the tulip bulb whose cells were analyzed with picoliter ESI MS. (A, B) Epidermal cells that occupy the two outermost cellular layers. (C, D) Parenchyma cells at a depth of 400 μm from the cuticle surface. Parenchyma cells, with their larger starch granules and a cube (or sphere) shape, can be distinguished from epidermal cells, with smaller starch granules and a rectangular solid shape. Scale bar = 100 μm for all panels (A–D).

MS analysis

After single-cell sampling and just before applying a high voltage, the outside of the pressure probe capillary (external capillary wall) was cleaned with an air duster. The pressure probe was flipped horizontally so that the quartz capillary tip was located close to the entrance (cone orifice) of the Orbitrap mass spectrometer at a distance of 5 mm. Thus, an atmospheric pressure ionization (API) method based on the electrification of the picoliter sample at the quartz capillary tip of the pressure probe was developed. The built-in ion source of an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Yokohama, Japan) was re-

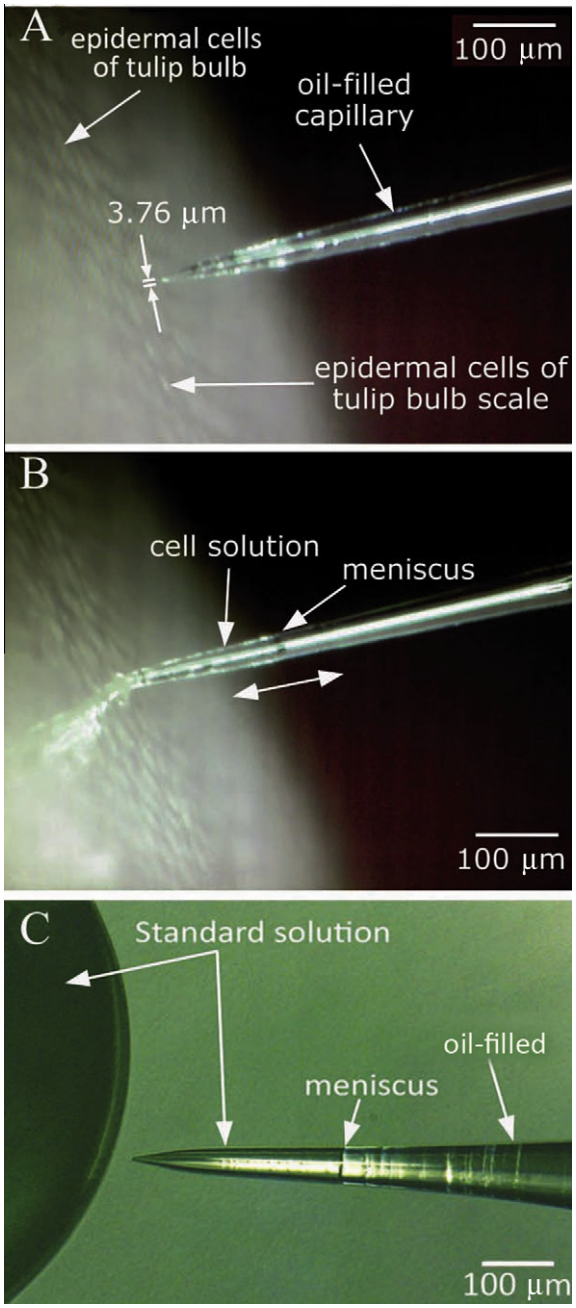

Fig.3. (A) The capillary tip is approaching a tulip bulb scale with the aid of a micrometer piezomotor. (B) The tip is penetrated into subepidermal parenchyma cells, and a meniscus is created at the interface of the cell solution and the oil mixture. (C) Standard compounds can be loaded on the pressure probe capillary tip from a 0.5-μl droplet hanged from a pipette tip.

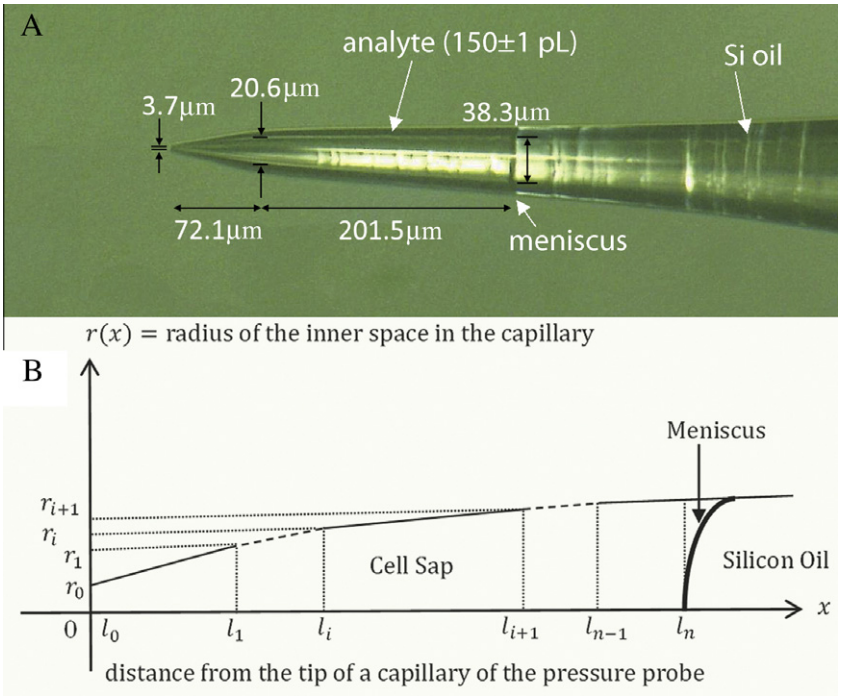

Fig.4. Calculation of cell sap volume. (A) Pressure probe capillary tip with picoliter cell sap sample or standard solution. (B) Parameters for calculation of the analyte solution volume at the pressure probe capillary tip (see Eqs. 1 and 2 in the Supplementary material).

placed with the pressure probe quartz capillary connected to a high-voltage generator. To maximize the sensitivity, automatic gain control (ion injection to the mass analyzer) was set to high dynamic range, the injection time was set to 250 ms, and the temperature of the sampling tube of the mass spectrometer was set to 200 °C. By applying +5 kV (Matsusada Precision, Japan) and −3 kV (ARIOS, Japan) to the tip, the analyte solution was directly nebulized and ionized (see Movie S-2 in Supplementary material), and no stream solvent or auxiliary gas spray was applied. An aqueous solution of glucose (40 mM), sucrose (40 mM), potassium citrate (10 mM), malic acid (10 mM), ascorbic acid (1 mM), nicotinic acid (1 mM), and hydroxyproline (1 mM), all chemicals from Wako Chemical (Japan), was prepared. Standard solution samples (100–200 pl) were loaded on the capillary tip with the aid of a pipette tip (Fig. 3C) and nebulized and ionized under high voltage. The acquired mass spectra were used as references for identifying possible peaks from the oil mixture or other contaminating compounds.

Mass spectra obtained by picoliter ESI MS analysis of the single-cell sap were compared with those from UV–MALDI MS and nano-ESI MS analyses. The methods used for UV–MALDI MS and nanoESI MS metabolite profiling of cell sap from tulip bulbs are fully explained in the Supplementary material.

Peak assignment

To verify the cell metabolite peaks with the aid of reference mass spectra, a list of monoisotopic masses of putative metabolites (with an accuracy of four decimal points) was created by the "Qual Browser" application of the Thermo Xcalibur software package (Thermo Fisher Scientific, Bremen, Germany). The mass values were compared with the exact mass values of the metabolites in several plant metabolite databases, including the "Plant Metabolite Network" (http://pmn.plantcyc.org), "SolCyc" databases (http://solcyc.solgenomics.net), "KNApSAcK" (http://kanaya.aist-nara.ac.jp/KNApSAcK/), and a list of metabolites from previous detection by MS of tulip bulb samples [3,11]. After matching mass values, chemical formulas of candidate metabolites were simulated by using isotope ratios in "Qual Browser" and then compared with the chemical formulas in the databases. The signal assignment of some metabolites was further confirmed by analyzing the tandem MS (MS/MS) fragmentation patterns of the signals compared with those of standard chemicals. The MS/MS analyses were carried out on a Thermo Scientific Velos Pro dual-pressure linear ion trap Orbitrap mass spectrometer. The collision-induced dissociation (CID) of parent species was induced with He at 35% collision energy. Fourier transform MS was used as the mass analyzer with a resolution of 7500 and a 1000-ms injection time. Spray voltages were +3 and −2.5 kV in positive and negative ion modes, respectively, with a capillary temperature of 275 °C.

Results and discussion

Sampling

The application of the cell pressure probe provided fully manageable sampling and handling of femtoliter to picoliter cell sap or standard solutions. In addition, the cell pressure probe facilitated accurate capillary localization, the absence of leakage (hydraulic continuity test; Movie S-1), and cell sample volume measurement. For quantitative single cell metabolomics, pressure probe facilitates the manipulation of the cell sap sample. In addition, because pressure probe can intrinsically measure cell turgor, it provides information about whether the target cell is intact or damaged. The type and location of the examined cell can also be distinguished by turgor pressure probing. For example, unlike phloem and parenchyma cells, xylem usually has negative tension. By recording the depth of penetration by the position indicator of a piezomotor manipulator system and with the anatomical information, the cell layer in which the capillary was located can also be determined.

In addition, because the pressure inside the capillary is accurately monitored and controlled, it is possible to both access and sample a deeper cell with minimal contamination from the shallower cell solution. In tulip bulbs, scale parenchyma cells act as

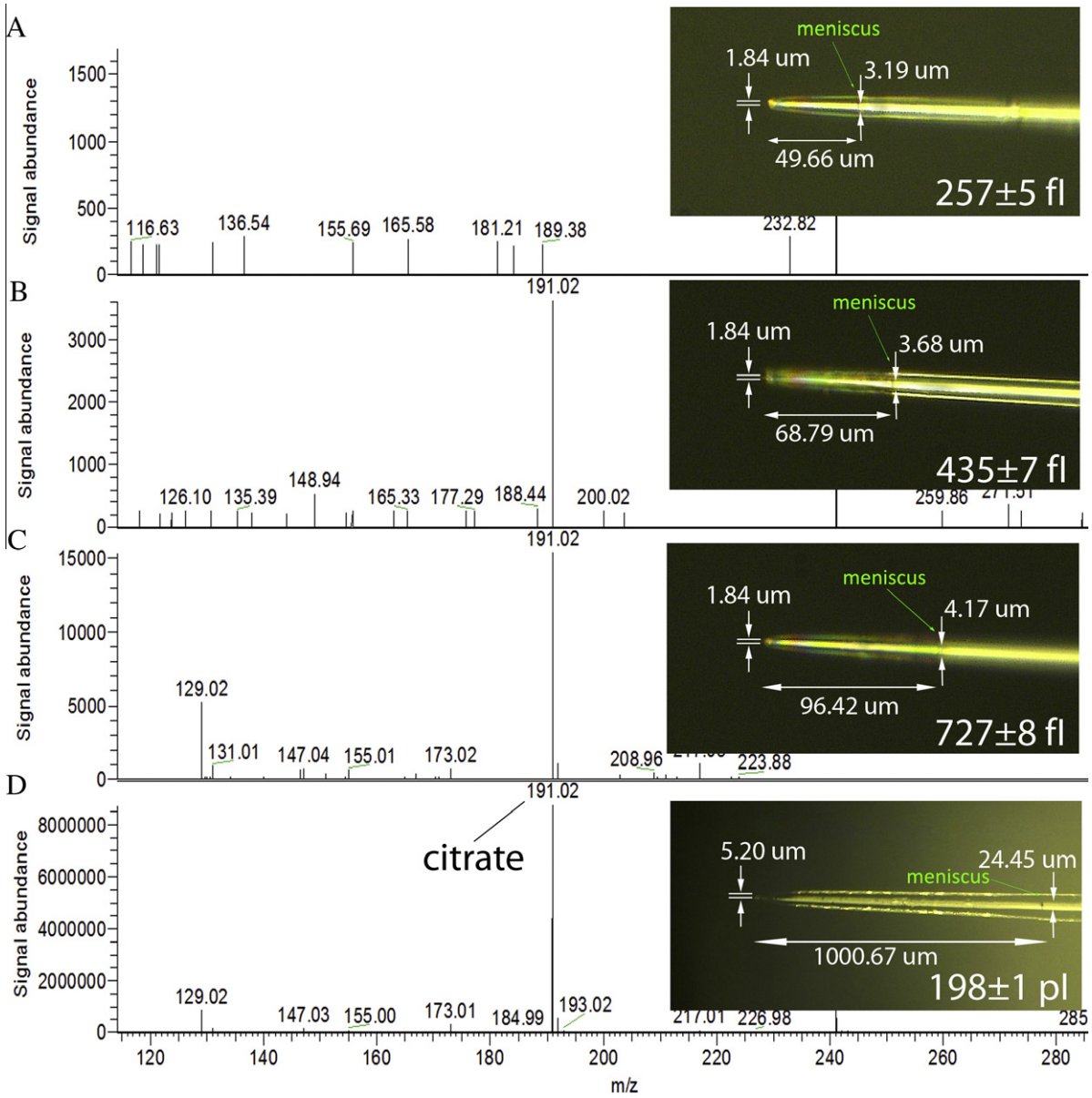

Fig.5. (A–C) Femtoliter samples of 257 fl (A), 435 fl (B), and 727 fl (C) were taken from a 500-mM citric acid solution (5% MeOH) by a cell pressure probe followed by direct electrospraying to an Exactive Orbitrap mass spectrometer. The signal of citrate appeared when 435 fl of solution was analyzed. (D) A picoliter solution of citric acid is sampled by another tip fabricated for cell sampling. For fabricating the capillary shown in panels A to C, higher laser heat with slower pulling speed was applied to generate a longer and narrower tip with a smaller opening. The difference in volume value due to the possible variation in contact angle ($45° < \theta < 90°$) of analyte solution at meniscus was considered as an error in volume calculation.

the source of energy, nitrogen, and carbon for the growing central flower stalk. In contrast, outer and inner epidermal cells of the bulb scales are usually small and contain smaller starch granules (Fig. 2). Therefore, in this work, we sampled and analyzed deeper parenchyma cells located below the second epidermal cells at a depth of approximately 400 μm from the scale surface. The average cell diameter was 100 μm, and depending on the size, water content, and turgor status of the cells, 20 to 800 pl of solution could be collected during each single-cell sampling. Cells of only a few micrometers in diameter (e.g., primary meristem cells of the tulip or parenchyma cells of some plants such as moss plants) may provide only sub-femtoliter sap samples for analysis. The pressure probe system, with a rod whose movement is controlled by a motorized micrometer, is able to manage femtoliter samples (Fig. 5). However, penetrating a capillary with a long and narrow tip specifically fabricated for femtoliter sampling into a plant cell with thick cell walls may pose a challenge. The selection of a proper material for capillary preparation is critical.

Ion source and signal acquisition

Because the common silicon oil (Si oil) used in the pressure probe is not electrically conductive, no analyte signals were detected from the cell sap and the aqueous standard solutions when using a pressure probe quartz capillary filled with Si oil. To increase the electrical conductivity for the electrification of the analyte solution in the capillary tip, an engine oil supplement (MolySpeed 21, Sumico, Japan) containing molybdenum and zinc organometalic macromolecules, among other additives, was added to the Si oil. Analyte signals appeared after adding a small amount of the supplement (0.1%) to the Si oil (Supplementary Fig. 1). As the percentage of the supplement in the oil mixture increased from

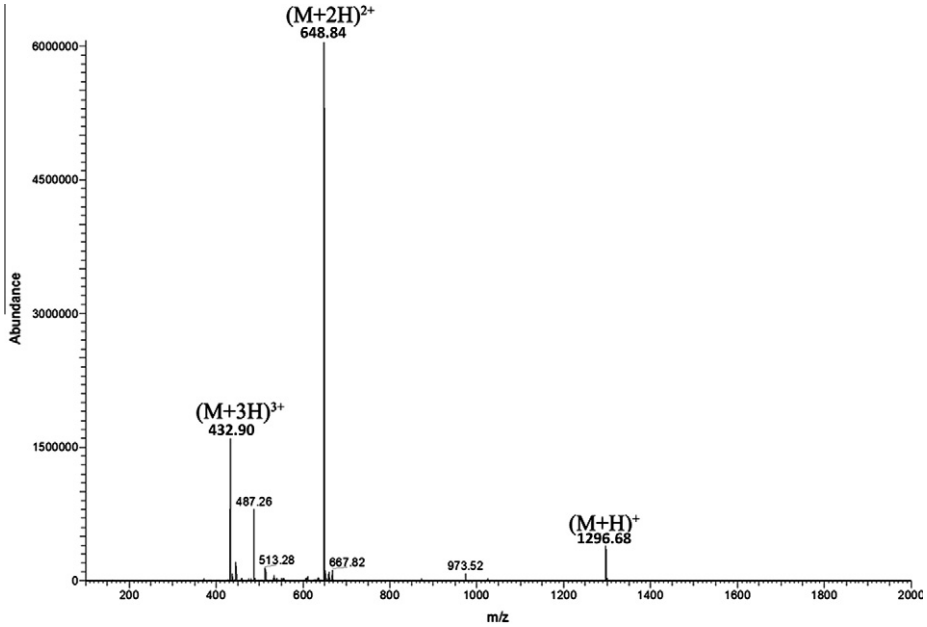

Fig.6. Picoliter ESI mass spectrum in positive ion mode of angiotensin I. Sample volume: 250 pl (40 mM aqueous solution).

0.1% to 1%, the abundance of oil-related signals also increased slightly. Likewise, the abundance of metabolite signals increased as the oil mixture ratio was raised to 1:9 (supplement oil 10%). The metabolite signals did not change significantly with a higher percentage of the supplement oil in the mixture. Therefore, in this work, 10% (1:9, v/v) was used for acquiring cell metabolite signals with a reasonable signal-to-noise ratio, intensity, and lower abundance of interfering signals.

Two approaches were considered for decreasing the abundance of interfering peaks. First, the oil mixture that accumulated outside of the capillary tip noticeably influenced the occurrence of contaminating signals. This oil load was nebulized and ionized freely and more easily than the analyte solution or the oil mixture inside the capillary tip (Supplementary Fig. 2). Therefore, cleaning the outside of the capillary with an air duster helped to reduce the abundance of interfering signals. Second, because the majority of the aqueous analyte solution did not nebulize/ionize at the same time as the oil mixture that was located either inside or outside of the capillary, a longer retention time with a higher abundance of cell metabolite signals and less abundant interfering signals could be selected in the chromatograms (Supplementary Fig. 3).

The ESI character of the picoliter ESI method is illustrated with the analysis of angiotensin I. The formation of multiply charged species is shown in Fig. 6. In the positive ion mode mass spectrum of the aqueous solution of this compound (angiotensin I, human, as acetate hydrate, Sigma), the intensity of the peaks for the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ species is higher than that for $[M+H]^{+}$. However, here there is no flow of the analyte solution, and unlike in common ESI and ESI-based ion sources such as nanoESI or recently developed PESI, the sample is ionized and introduced into the mass spectrometer without the addition of a stream solvent or auxiliary gas (Movie S-2). In positive ion mode, at lower voltage (+3 kV), a large solution droplet appeared at the tip (see the first part of Movie S-2), showing that the voltage applied was not enough to overcome the surface tension, although ionization was still taking place. By applying +5 kV to the capillary pressure probe tip, ionization improved (see second part of Movie S-2) and the 250-pl solution nebulized and ionized immediately at the tip of the capillary without droplet formation. When changing the voltage in the range of +3 to +5 kV, the abundance of the analyte signals did not change significantly. In negative ion mode, the application of −3 kV was enough for the nebulization/ionization of the picoliter cell sap or standard solution in the capillary tip. The pressure probe instrument was located close to a mass spectrometer, and the metabolite composition of the sample was analyzed immediately after sampling and taking photos of the capillary tip for the volume calculation. Therefore, the possibility of loss, contamination, or evaporation of the sample was minimized. The complete nebulization/ionization of picoliter cell samples usually occurred within a few seconds.

Femtoliter solutions can also be analyzed; a signal from the citric acid standard was detected with the analysis of approximately 435 fl of a 500-mM aqueous solution (Fig. 5B). Difficulty in the electrospray process of a femtoliter solution from a tip with an opening of approximately 1 μm arises due to the surface tension. By adding a small percentage of methanol (i.e., 5%) and, if needed, raising the oil pressure inside capillary, the surface tension can be overcome and the analyte solutions can then be nebulized/ionized more efficiently. However, as a result of the stronger surface tension at the tip, the femtoliter ESI signal yield would be less than the picoliter ESI signal yield. Consequently, the limit of detection (LOD) was higher when analyzing femtoliter solutions (~218 fmol; Fig. 5) compared with picoliter solutions (with an LOD of 30 fmol; Table 1). For the MS metabolomic analyses of plant single cells that have a greater number of metabolites coexisting in the mixture, picoliter solutions may be needed. The difficulty in the detection of some metabolites when analyzing femtoliter cell sap samples may originate from their very low natural concentrations. In addition, the LOD of metabolites can change when they are analyzed in a mixture. For example, approximately 0.03 pmol (30 fmol) of citric acid was detected in a pure solution (Table 1), but the LOD of citric acid in a mixture of seven compounds was approximately 13 pmol (Table 1, negative ion mode). When analyzing mixture fluidic samples, ionization of each analyte is influenced by the existence of other molecules. This so-called ion suppression phenomenon [27] caused by the matrix or compounds present in the mixture is one of the major difficulties in ESI MS [28]. Nevertheless, in negative ion mode, fewer analytes respond to high voltage and, consequently, target metabolites may be subjected to less ion suppression [28].

Table 1
LODs (pmol) of metabolite standards analyzed by picoliter ESI MS.

Standard	Pure standard		Standard in the mixture	
	Negative ion mode	Positive ion mode	Negative ion mode	Positive ion mode
Ascorbic acid	0.04	32.0	3.0	ND
Citric acid	0.03	3.4	13.0	330.0
Glucose	0.11	0.5	19.0	39.0
Hydroxyproline	0.04	3.0	54.0	54.0
Malic acid	0.50	162.0	52.0	ND
Nicotinic acid	0.03	3.0	52.0	34.0
Sucrose	0.50	1.2	39.0	39.0

Note: The mixture solution was composed of glucose (40 mM), sucrose (40 mM), potassium citrate (10 mM), malic acid (10 mM), ascorbic acid (1 mM), nicotinic acid (1 mM), and hydroxyproline (1 mM) diluted with water. ND, not detected.

The lower limits of the volume for the detection of metabolites in single-cell samples from tulip bulbs were 25 and 60 pl in negative and positive ion modes, respectively. Table 1 shows the LOD of a number of standard chemicals in a mixture whose signals could be detected in cell solution samples. Potassium was added to the mixture in the form of potassium citrate to simulate the natural abundance of potassium in the cell sap. The LOD of the examined metabolites was not necessarily similar and varied between 0.03 and 54 pmol in negative ion mode and between 0.5 and 330 pmol in positive ion mode. To examine the reproducibility of the signal acquisition with this technique, 12 cells located in a small area on the second scale of a cooled bulb were consecutively sampled, and their metabolite contents were profiled. Supplementary Fig. 4 shows the signal abundance of 38 metabolites in picoliter samples from those cells. Despite the natural cell-to-cell variations in the abundance of metabolites, the total signal abundance of metabolites in examined cells was not significantly different (Supplementary Fig. 4B) and represents the overall metabolite content in cells with a similar phenotype.

We compared the mass spectra of the cell sap with those of a mixture of standard metabolites. The signals that originated from the quartz capillary material, Si oil, engine supplement, and other sources were distinguished from the peaks of the cell sap components. The interfering clusters appeared only when the cell sap or standard aqueous solutions were present in the pressure probe capillary tip. After loading an aqueous solution to the tip, a series of signals in the m/z region of 400 to 700 with 74 m/z units of separation appeared in the positive ion mode (Supplementary Fig. 3). This repeating unit can be attributed to the replacement of the [–OH] functional groups by [–OSi(CH3)$_3$] (trimethylsiloxane) groups, a common end-capping function [29].

Metabolite profiling of tulip bulb cells

The detection of carbohydrates (hexose, sucrose, and high-molecular-weight soluble carbohydrates) in tulip bulb cell sap with UV–MALDI MS [11] and the detection of carbohydrates together with arginine and tuliposide A in tulip bulb tissue samples with PESI MS [3] have been reported previously. In the current work, for the purpose of comparison, UV–MALDI MS and nanoESI MS analyses of tulip bulb single-cell sap were conducted (Supplementary Figs. 5 and 6). The results of nanoESI MS metabolite profiling of cell sap were used for comparison and confirmation of the assignment of peaks that were detected (Supplementary Figs. 7–22). With this technique, a large number of metabolites belonging to the sugar and amino acid metabolic pathways, central respiration, Krebs cycle, lipid metabolism, and secondary metabolites were identified in both positive ion mode (Fig. 7A and Supplementary Figs. 8–13) and negative ion mode (Fig. 7B and Supplementary

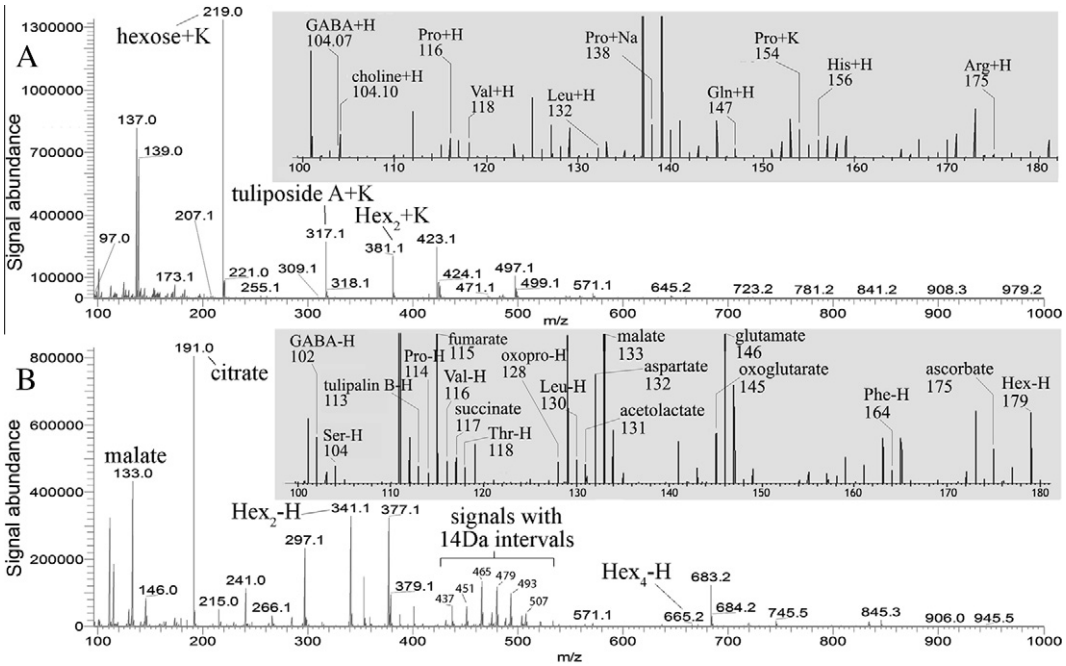

Fig.7. Picoliter ESI mass spectra acquired with 100-pl samples from parenchyma cells of a second scale of a cooled tulip bulb in positive (A) and negative (B) ion modes. Insets are the magnifications of m/z 100–180 and m/z 100–180 regions of the mass spectra shown in panels A and B. See Supplementary Figs. 8–22 for details.

Figs. 14–22). Supplementary Table 1 lists the detected metabolites. MS/MS mass spectra of the selected metabolites are shown in Supplementary Figs. 23–33.

Succinic, fumaric, oxaloacetic, malic, and citric acids, whose signals were detected in single-cell sap samples, are important intermediates in the Krebs cycle. Among the sugars, monohexose (Hex) to pentahexose (Hex_5) and ascorbic acid, a secondary product of the sugar metabolism, were detected. From the amino acid metabolic pathways, 13 proteinogenic amino acids (Val, Leu, Pro, Gln, His, Arg, Trp, Ala, Ser, Thr, Asp, Glu, and Phe), choline, and oxoproline were identified. Aminobutyric acid (GABA), which has a key role in the response to stress and in carbon/nitrogen metabolism [30], and putrescine, a polyamine that has been speculated to have important roles in cell growth [31] and in the response to different abiotic stresses in plants [32], were also detected. Among the secondary metabolites, we identified tulipalin A, tulipalin B, and tuliposide A. Among the intermediates in lipid metabolism, lauric, linoleic, myristic, oleic, palmitic, and stearic acids, together with glyoxilic acid (which links lipid metabolism to the Krebs cycle), were also detected. A number of peaks with 14 units of *m*/*z* intervals appeared in the negative ion mode mass spectra (Fig. 7B and Supplementary Fig. 21). This repeating unit can be attributed to alkane chains, typical of lipidic compounds with the moiety ($-[CH_2]_n-$) in their structure [29].

One of the features of the pressure probe is that it facilitates the handling of femtoliter to picoliter solutions of standard chemicals. After cell sampling, it is possible to load the standard chemicals into the capillary tip. The added chemical(s) then nebulizes/ionizes together with cell metabolites and can be used as an internal calibrant for mass calibration or quantitation purposes. In our work, we used mannitol as an internal standard because we did not detect its signal in tulip cell samples. Because the same volume of standard mannitol (150 pl) was added to 100 pl of cell sap sucked from both the control and cooled bulbs, the signal intensities reflected the natural changes of those metabolites due to the influence of low temperature during storage of the bulbs. In Fig. 8, the abundance of sucrose (Hex_2), hexose, and tuliposide A in the control and cooled bulbs is shown, with the potassiated mannitol peak as the reference. It has been shown that low temperature induces starch degradation in bulbs, which results in the accumulation of underivatized sugars (hexose, sucrose, and soluble fructans) in the scale cells [33–35]. A significant difference was observed in the abundance of hexose and sucrose in the control and cooled bulb cells (Fig. 8). These results are consistent with those of our previous in situ UV–MALDI MS tissue analyses of tulip bulbs [36]. In addition, a higher abundance of tuliposide A was observed in the cooled bulb cell sap (Fig. 8B). As a natural antibiotic, tuliposide A accumulates in tulip bulbs to strengthen the cellular defense against potential diseases in the cool humid storage environment [37–39]. Tuliposide A is also a substrate for the biosynthesis of tulipalins, which are another group of abundant antibiotics in tulip bulbs [40] whose concentration increases in response to the attack of fungi such as *Fusarium oxysporum* [41].

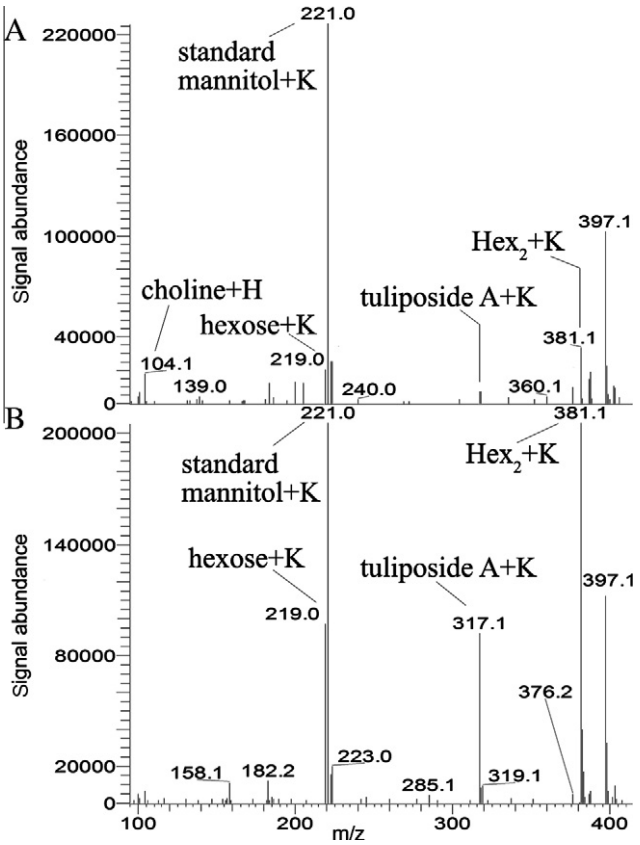

Fig.8. Picoliter ESI mass spectra of 100-pl sap samples sucked from single cells located at second scales of a control (A) and a cooled tulip bulb (B). As an internal reference, 150 pl of an aqueous 100-mM mannitol solution was added to the cell sap sample inside the pressure probe capillary tip.

Conclusions

In this work, we have reported the online application of a cell pressure probe and an Orbitrap mass spectrometer for in situ single-cell sampling and metabolome analysis in real time. Accurate localization of the quartz capillary tip in a target cell, sample volume measurement, deep cell sampling with minimal contamination from other cells, and precise sub-femtoliter solution management are unique features of the pressure probe. Immediate nebulization/ionization of cell samples with minimal sample loss provided a great opportunity for rapid metabolite profiling. Some important prerequisites for quantitative metabolomics of single cells (i.e., the measurement of the volume of the cell sap sample and the addition of internal reference or calibrants to analyte solutions) are fulfilled by this technique. Before sampling, it is possible to use the pressure probe for cell osmotic and water potentials, turgor, plasma membrane hydraulic conductivity, and cell wall elastic modulus measurements, which, together with metabolome analyses using the pressure probe as an ion source, can provide comprehensive insights into the function and behavior of cell molecule components during growth or stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.10.001.

References

[1] A. Amantonico, P.L. Urban, R. Zenobi, Analytical techniques for single-cell metabolomics: State of the art and trends, Anal. Bioanal. Chem. 398 (2010) 2493–2504.

[2] K. Hiraoka, K. Nishidate, K. Mori, D. Asakawa, S. Suzuki, Development of probe electrospray using a solid needle, Rapid Commun. Mass Spectrom. 21 (2007) 3139–3144.

[3] Z. Yu, L.C. Chen, H. Suzuki, O. Ariyada, R. Erra-Balsells, H. Nonami, K. Hiraoka, Direct profiling of phytochemicals in tulip tissues and in vivo monitoring of the change of carbohydrate content in tulip bulbs by probe electrospray ionization mass spectrometry, J. Am. Soc. Mass Spectrom. 20 (2009) 2304–2311.

[4] H. Mizuno, N. Tsuyama, T. Harada, T. Masujima, Live single-cell video–mass spectrometry for cellular and subcellular molecular detection and cell classification, J. Mass Spectrom. 43 (2008) 1692–1700.

[5] N. Tsuyama, H. Mizuno, E. Tokunaga, T. Masujima, Live single-cell molecular analysis by video–mass spectrometry, Anal. Sci. 24 (2008) 559–561.

[6] M.L. Tejedor, H. Mizuno, N. Tsuyama, T. Harada, T. Masujima, Direct single-cell molecular analysis of plant tissues by video mass spectrometry, Anal. Sci. 25 (2009) 1053–1056.

[7] B. Shrestha, A. Vertes, In situ metabolic profiling of single cells by laser ablation electrospray ionization mass spectrometry, Anal. Chem. 81 (2009) 8265–8271.

[8] H. Nonami, J.S. Boyer, Wall extensibility and cell hydraulic conductivity decrease in enlarging stem tissues at low water potentials, Plant Physiol. 93 (1990) 1610–1619.

[9] S. Kajiyama, K. Harada, E. Fukusaki, A.J. Kobayashi, Single cell-based analysis of *Torenia* petal pigments by a combination of ArF excimer laser micro sampling and nano-high performance liquid chromatography (HPLC)–mass spectrometry, Biosci. Bioeng. 6 (2006) 575–578.

[10] Y. Izumi, S. Kajiyama, R. Nakamura, A. Ishihara, A. Okazawa, E. Fukusaki, Y. Kanematsu, A. Kobayashi, High-resolution spatial and temporal analysis of phytoalexin production in oats, Planta 229 (2009) 931–943.

[11] Y. Gholipour, H. Nonami, R. Erra-Balsells, Application of pressure probe and UV–MALDI–TOF MS for direct analysis of plant underivatized carbohydrates in subpicoliter single-cell cytoplasm extract, J. Am. Soc. Mass Spectrom. 19 (2008) 1841–1848.

[12] Y. Gholipour, R. Erra-Balsells, H. Nonami, In situ pressure probe sampling and UVMALDI MS for profiling metabolites in living single cells, Mass Spectrom. 1 (2012) A0003.

[13] R. Erra-Balsells, Y. Gholipour, H. Nonami, In situ pressure probe sampling of single cell solution from living plants for metabolite analyses with UV–MALDI MS, Lecture Notes Eng. Comput. Sci. 2197 (2012) 572–577.

[14] P.J. Kramer, J.S. Boyer, Cell water relations, in: Water Relations of Plants and Soils, Academic Press, San Diego, 1995, pp. 42–83.

[15] J.S. Boyer, Pressure probe, in: Measuring the Water Status of Plants and Soils, Academic Press, San Diego, 1995, pp. 103–142.

[16] E. Hüsken, E. Steudle, U. Zimmermann, Pressure probe technique for measuring water relations of cells in higher plants, Plant Physiol. 61 (1978) 158–163.

[17] K.A. Shackel, Direct measurement of turgor and osmotic potential in individual epidermal cells, Plant Physiol. 83 (1987) 719–722.

[18] H. Nonami, E.D. Schulze, Cell water potential, osmotic potential, and turgor in the epidermis and mesophyll of transpiring leaves: combined measurements with the cell pressure probe and a nanoliter osmometer, Planta 177 (1989) 35–46.

[19] E. Steudle, U. Zimmermann, Effect of turgor pressure and cell size on the wall elasticity of plant cells, Plant Physiol. 59 (1977) 285–289.

[20] P.B. Green, R.O. Erickson, J. Buggy, Metabolic and physical control of cell elongation rate: In vivo studies in *Nitella*, Plant Physiol. 47 (1971) 423–430.

[21] M. Malone, A.D. Tomos, A simple pressure-probe method for the determination of volume in higher-plant cells, Planta 182 (1990) 199–203.

[22] J. Zeleny, Instability of electrified liquid surfaces, Phys. Rev. 10 (1917) 1–6.

[23] M. Mann, Electrospray: Its potential and limitations as an ionization method for biomolecules, Org. Mass Spectrom. 25 (1990) 575–587.

[24] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, Science 246 (1989) 64–71.

[25] M.S. Wilm, M. Mann, Electrospray and Taylor–Cone theory: Dole's beam of macromolecules at last?, Int J. Mass Spectrom. Ion Proc. 136 (1994) 167–180.

[26] M. Wilm, M. Mann, Analytical properties of the nanoelectrospray ion source, Anal. Chem. 68 (1996) 1–8.

[27] L.L. Jessome, D.A. Volmer, Ion suppression: a major concern in mass spectrometry, LCGC N. Am. 24 (2006) 498–510.

[28] D.L. Buhrman, P.I. Price, P.J. Rudewicz, Quantitation of SR 27417 in human plasma using electrospray liquid chromatography–tandem mass spectrometry: a study of ion suppression, J. Am. Soc. Mass Spectrom. 7 (1996) 1099–1105.

[29] H. Tong, D. Bell, K. Tabei, M.M. Siegel, Automated data massaging, interpretation, and e-mailing modules for high throughput open access mass spectrometry, J. Am. Soc. Mass Spectrom. 10 (1999) 1174–1187.

[30] N. Bouché, H. Fromm, GABA in plants: just a metabolite?, Trends Plant Sci 9 (2004) 110–115.

[31] C. Wang, J. Delcros, L. Cannon, F. Konate, H. Carias, J. Biggerstaff, R.A. Gardner, O. Phanstiel, Defining the molecular requirements for the selective delivery of polyamine conjugates into cells containing active polyamine transporters, J. Med. Chem. 46 (2003) 5129–5138.

[32] R. Alcăzar, J. Planas, T. Saxena, X. Zarza, C. Bortolotti, J. Cuevas, M. Bitriăn, A.F. Tiburcio, T. Altabella, Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous *Arginine decarboxylase 2* gene, Plant Physiol. Biochem. 48 (2010) 547–552.

[33] R. Moe, A. Wickström, The effect of storage temperature on shoot growth, flowering, and carbohydrate metabolism in tulip bulbs, Physiol. Plant. 28 (1973) 81–87.

[34] E. Haaland, A. Wickström, The effect of storage temperature on carbohydrate interconversion in tulip bulbs, Acta Hortic. 47 (1975) 371–376.

[35] H. Lambrechts, F. Rook, C. Kolloffell, Carbohydrate status of tulip bulbs during cold-induced flower stalk elongation and flowering, Plant Physiol. 10 (1994) 515–520.

[36] Y. Gholipour, S.L. Giudicessi, H. Nonami, R. Erra-Balsells, Diamond, titanium dioxide, titanium silicon oxide, and barium strontium titanium oxide nanoparticles as matrixes for direct matrix-assisted laser desorption/ionization mass spectrometry analysis of carbohydrates in plant tissues, Anal. Chem. 82 (2010) 5518–5526.

[37] R. Tschesche, F.-J. Kämmerer, G. Wulff, F. Schönbeck, Über die antibiotisch wirksamen substanzen der Tulpe (*Tulipa gesneriana*), Tetrahedron Lett. 9 (1968) 701–706.

[38] R. Tschesche, F.-J. Kämmerer, G. Wulff, Über die struktur der antibiotisch aktiven substanzen der tulpe, Chem. Ber. 102 (1969) 2057–2071.

[39] L.P. Christensen, Tuliposides from *Tulipa sylvestris* and *T. turkestanica*, Phytochemistry 51 (1999) 969–974.

[40] F. Schönbeck, C. Schroeder, Role of antimicrobial substances (tuliposides) in tulips attacked by *Botrytis* spp, Physiol. Plant Pathol. 2 (1972) 91–99.

[41] J.C. Beijersbergen, C.B.G. Lemmers, Enzymatic liberation of tulipalin (α-methylenebutyrol-actone), a fungitoxic substance isolated from tulips, Acta Hort. 23 (1971) 230–234.