

Integrative Analysis of Physiological Phenotype of Plant Cells by Turgor Measurement and Metabolomics

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Abstract—Water status and metabolite content are considered as two key features in plant cell physiological phenotype. In order to profiling *in situ* living plant cell status, turgor pressure of cells located at different locations of tissues was probed with a cell pressure probe and then cell sap was sampled and its metabolite profile was generated with using nanoESI and MALDI mass spectrometry. No purification or separation was included in workflow and picoliter cell sap samples were injected directly into a nanoESI-Orbitrap mass spectrometer and/or deposited on selected matrices from organic compounds and nanoparticles for MALDI-TOF mass spectrometry analysis. Both shotgun mass spectrometry techniques could be used for detecting and quantifying metabolites in single-cell samples. Different metabolites from neutral carbohydrates to amino acids and secondary metabolites could be detected. Quantity of two major metabolites, sucrose and kestose, was also measured in several cells and sucrose concentration was co-plotted with turgor data.

Index Terms—metabolite profiling, MALDI, nanoESI, tulip

I. INTRODUCTION

INFORMATION on the water status and on the content of metabolites obtained by tissue-level analysis, so-called bulk biochemistry [1], does not necessarily reflect cellular physiological phenotype since physiological status may vary in different parts of cells (e.g. in cytoplasm and apoplast), among cells in a particular tissue (cell to cell variations in different location of tissue) and at same cells during developmental or environmental events. Additionally, this type of study is time-resolved and necessarily cannot be used for analyzing intracellular events in real time. On the other hands, integrative studies which include joint analyses of several cell features have advantage of providing more

comprehensive insights about cellular life. Modern biology research focus, therefore, is moving from classical one-dimension organ or tissue-level analyses to integrative single-cell biology. In this context, combinational plant water status measurement and omics analyses with the single-cell resolution can explore basic aspects of life, cell to cell variations, primary responses to abiotic stresses or biotic attacks, and the processes of growth or death. For such a molecular analysis of single cells, a reliable access to the cell solution, the sampling in real time, and a strong detection power are critical. Time-resolved sampling metabolites of single cells from fixed tissues have been successfully illustrated by using laser micro-dissection [2]–[4]. A transparent glass capillary tube attached to an oil-filled pressure probe is commonly used to measure turgor of living plant cells [5]. Application of the pressure probe, which is commonly used for measuring turgor of *in situ* cells, for sampling cells will also have several advantages to a simple capillary, all originating from the fact that the hydrostatic pressure inside the capillary can be managed [6]; amount of cell sap to be extracted can be accurately controlled; inward and outward movement of sap inside the capillary will become possible; and more important and inimitably, the capillary can pass through several cell layers to get the target cell layer deep in the tissue while entering the cytoplasm of cells on the way until reaching the target cell layer can be avoided. Accordingly, the pressure probe has been used for measuring turgor pressure and sampling cytoplasm of single cells located in different parts of plant tissues [6]–[12] and is a great candidate for integrative cellular studies.

Modern mass spectrometers equipped with soft ionization and high sensitive detectors are able to detect minute quantities of biomolecules. Classically, biomolecules are extracted from biological tissues, separated, purified and finally analyzed by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). A high-throughput separation-free (i.e. shotgun) analysis, has attracted considerable attention. Recently, MALDI mass spectrometry has been used for analyzing sub-picoliter volume of single-cell samples [6]. On other hands, mass spectrometry was introduced for quantification of biomolecules in early years of its development [13], [14] and nowadays is turning to a well-established technique for quantitative analysis in proteomics [15]. For quantitation of metabolites in femto- to picoliter volume of cell samples, samples must be carefully handled and measured. Otherwise,

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the interpretation of the signal abundance as the relative natural change of metabolite content in cells would not be reliable.

Here we report the application of a cell pressure probe for reliable accessing, turgor pressure probing and sampling of living plant cells followed by identification and quantification of metabolites by using nanoESI-Orbitrap and MALDI-TOF mass spectrometry. No purification or separation step was included in the workflow. For nanoESI-MS analysis, cell sap of picoliter sample was directly injected into the ion source and for MALDI-MS cell sample was deposited on selected matrices. MALDI-MS quantitation of sugars was carried out with titanium dioxide nanoparticles (NPs) which have been shown to be efficient matrix for quantitation of picomoles of soluble underivatized carbohydrates [16].

II. MATERIALS & METHODS

A. Cell Turgor Measurement

The cell pressure probe consisted of a microcapillary connected to a pressure transducer (XTM-190M-100G, Kulite Semiconductor Products Inc., USA), a piezo motor (PM101 Märzhäuser Wetzlar, Germany) mounted on a 3D micro-manipulator, a motorized micrometer having a rotational metal rod, and its speed controller. By rotating the micrometer with a speed-adjustable motor (Oriental Motor Co. LTD, Japan), changes in silicon oil volume in the pressure probe could be adjusted. To minimize the vibrations, pressure probe and its accompanying instruments were placed on a magnetically-floated table. The capillary tip manipulation was accurately performed. The operation was monitored under a digital microscope (VHX-900 digital microscope, Keyence Co., Osaka, Japan) which facilitated recording the experiment and an online measurement of the sample volume. The pressure transducer was connected to a digital pressure display and a chart recorder. The pressure probing process was, therefore, monitored and recorded for further data analysis.

Anatomy of the second scale tulip bulb was previously studied and hence, the location and the depth of the target cells were known (Fig. 1). With a 3D manipulator the capillary tip was located and penetrated by pushing the tip into the tissue by the piezo-motor. The penetration depth was monitored since a distance in the horizontal movement of the probe tip was displayed in the controller of the piezo-motor. Quartz capillaries were used and tapered with a laser-heated micropipette capillary puller (P-2000 Sutter Instrument). The pressure probe microcapillary tip (about $2.5\ \mu\text{m}$ O.D.) was used for probing tulip scale cells with a diameter of about $100\ \mu\text{m}$ and the volume of about $1\ \text{nL}$.

In normal plant cells, hydraulic pressure is formed by turgor pressure which leads to an elastic expansion of the cell wall and with the cell volume maintained [17]–[20]. After penetration of the tip into a cell, the cell solution entered into the capillary tip due to the turgor pressure of the cell [19]–[21]. Since the cell solution was not soluble in silicon oil, two phases with a meniscus at the interface appeared (Fig.

2A). Both water and oil are incompressible, and since the whole system from the pressure transducer to the capillary tip was assembled so as to be air-tight, a sub-millibar change inside capillary tip was immediately sensed by the pressure transducer far from the capillary. On the other hand, pushing the rod inside the tubing using a micrometer connected to the speed-control motor correspondingly led to a forward movement of silicon oil and a backward movement of cell solution. Since the diameter of the rod was $0.4\ \text{mm}$ and the movement was in the nanometer-order, a sub-picoliter volume of oil or sample solution could be handled conveniently. In practice, with the $0.4\ \text{mm}$ radius of the rod and varying the viscosity and the surface tension of aqueous standard or cell solutions, $1\text{--}10\ \text{pL}$ volume inside the tip could be handled.

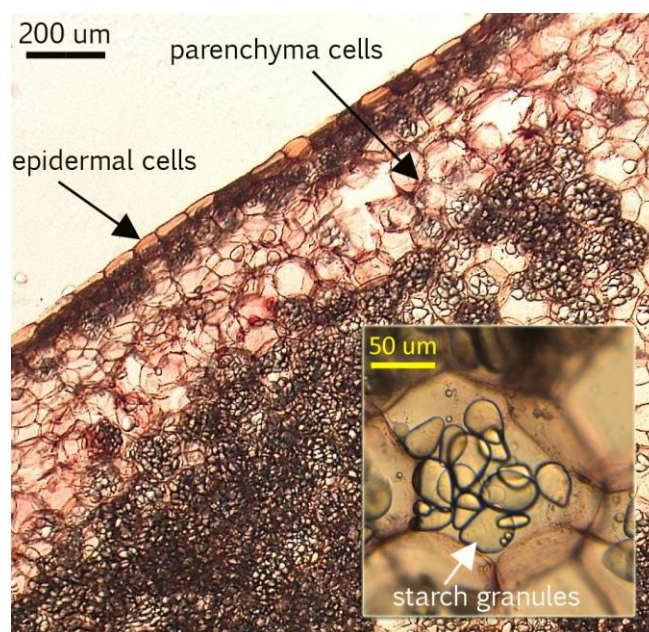


Fig. 1. Photo of the cross-section of the second scale of a tulip bulb; the location of parenchyma cells with abundant starch is shown. Parenchyma cells located under epidermis down to the depth of around $600\ \mu\text{m}$ were accessed and analyzed by using a cell pressure probe. Inset in the right-bottom of the figure magnifies one of the target parenchyma cells with abundant big starch granules.

Before probing turgor pressure, a hydraulic continuity test [20] was performed. Pressure was decreased and increased quickly several times and if the meniscus could be moved back and forth correspondingly, it would show a perfect hydraulic connection between the target cell and the pressure transducer. Additionally, while the tip has been already inserted into the inside of a cell, the pressure was kept unchanged for a period and if the pressure and the meniscus location remained unchanged, it confirmed that no cell solution has leaked. A full detail of pressure probe set-up, operation and measurements can be found in our comprehensive review on the application of this technique to integrative plant cell analyses [22].

C. Single-Cell Sampling

Turgor pressure caused the entering of $10\text{--}600\ \text{pL}$ (varying with cell size, tissue type, and cell water status) of cell

cytoplasm into the microcapillary. After measuring turgor of a target cell located at a known depth, the capillary tip was taken out of the tissue while the oil pressure was controlled, in order to preserve the sample inside the tip. The dimensions of the cell sample were immediately measured under the microscope, the volume of the truncated cone-shaped sample was calculated, and the tip with the sample inside was photographed to record the experiment. The volume of the cell sap inside the capillary tip can be determined by measuring the radius with respect to a distance from the tip up to the location of the meniscus [22], [23]. By balancing the pressure inside the capillary against the atmospheric pressure, the surface tension at the tip could hold the cell sap in the capillary. The tip was then inserted into a micropipette containing 0.5 μL of water followed by transfer of extract into the pipette by applying positive pressure in the microcapillary (Fig. 2B). The change in the volume of water droplet inside the pipette tip is negligible for about 10 minutes at room temperature [22]. On other hand, the whole process of injecting a picoliter sample into nanoESI ion source or transferring the sample into the water droplet and depositing a droplet of the mixture of the sample and the water droplet on a previously air-dried matrix layer on the plate could be carried out within 1-3 minutes.

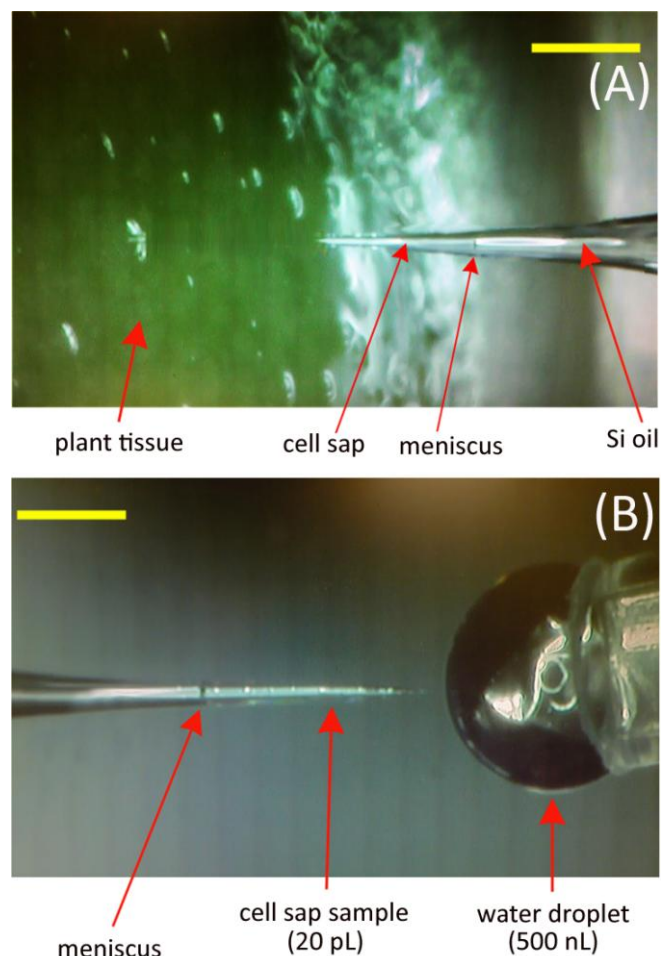


Fig. 2. (A) Photo of a capillary of the pressure probe which was inserted in a cell, having the meniscus between cell sap and the silicon oil (scale bar = 400 μm). (B) Injection of cell microsample into water droplet facilitated its transfer for mass spectrometry analyses (scale bar = 400 μm).

D. Materials and Preparations

Tulip (*Tulipa gesneriana* L.) bulbs were supplied by a local grower.

2,4,6-Trihydroxyacetophenone (THAP) was purchased from Fluka (St. Gallen Buchs, Switzerland) and sucrose (ultra-pure and 1-kestose (>99% purity) from Wako Chemicals (Osaka, Japan). HPLC grade methanol (MeOH) (Merck, Darmstadt, Germany) was used without further purification. Titanium dioxide NPs (DP-25-nm diameter with 80% anatase and 20% rutile) was obtained from Evonik Degussa Corp., USA. Water with very low conductivity of Milli-Q grade that was purified at 56-59 nS/cm with a PURIC-S, (Orugano Co., Ltd., Tokyo, Japan) was used. Saturated solution of THAP in acetone was prepared. Aqueous mixture solutions of standard sucrose and kestose were prepared.

E. Mass Spectrometry Analysis

A cell sap sample in 500 nL water droplet was injected into the ESI ion source by using a 0.5 μL Hamilton syringe and a Rheodyne tee inserted at the distance of 20 cm from ion source. Nano-ESI-MS Analysis was carried out on Thermo Exactive™ Orbitrap mass spectrometer with aqueous MeOH 50% as spraying solvent (5 $\mu\text{L}\cdot\text{min}^{-1}$) and 3 kV spraying voltage. For MALDI-MS analysis 500 nL analyte solution was deposited on an air-dried layer of titanium dioxide NPs previously deposited on MALDI plate and the analysis was carried out on an AB SCIEX TOF/TOFTM 5800 System (Framingham, MA 01701, USA) having a 349-nm Neodymium-doped yttrium lithium fluoride laser. To obtain good resolution and signal-to noise (S/N) ratios, the laser power was adjusted to slightly above the threshold, and each mass spectrum was generated by averaging 100 lasers pulses per spot. For each sample spot on the plate, 15 tiles each 400×400 μm^2 , 10 at the edge and 5 at the center were determined. Each tile is shot with 400 laser pulses and is separately saved. Regarding the dimension and the distribution of the dried cell sample on the plate and the size of the tile, it seemed 1-2 tiles would be enough to localize all cell sample aggregates. Those mass spectra with cell metabolite signals were used for further analyses and reporting the experiment. Since the number of molecules of cell metabolites would be in the range of attomoles to picomoles, therefore, after 400 laser shots, almost all of the cell metabolites located at irradiation area seemed to desorb and ionize. Peaks of mass spectra with exact mass acquired with nanoESI- and MALDI-MS were assigned to metabolites reported to be detected in tulip in (KNAPSAcK®, RIKEN, Japan) and plant metabolic network (www.plantcyc.org). For quantitation, 500 nL of aqueous standard sucrose and kestose mixture solutions was used.

III. RESULTS & DISCUSSION

The target cells of this study were parenchyma cells located at different location at the depth of 100-600 μm from the cuticle of the second scale of cooled tulip bulbs (Fig. 1). A

primary anatomical study helped select the location and penetration depth to acquire cell sap sample from suitable parenchyma cells located in storage tissues of the bulb. Those cells store abundantly starch granules from which soluble underivatized sugars are produced specially after bulbs are exposed to low temperature [24], [25]. Managing the location of the meniscus and measuring oil pressure inside capillary are core operations in pressure probe technique. For example, the pressure needed to return the meniscus to its original position where it is before the tip had penetrated the cell, is equal to turgor of that cell. In addition to the cell turgor, several other properties of a cell can be measured with the pressure probe including cell wall elastic modulus [26], [27], cell wall extensibility [26], hydraulic conductivity [7], [28], [29] of the plasma membrane, and the volume of the target cell [30]. A part of the cell solution sample inside the capillary tip can be transferred to a picoliter cryo-osmometer plate and subsequently, the osmotic potential of the cell is directly measured with a picoliter cryo-osmometer [31], [32]. Water potential is then uniquely calculated with a single-cell resolution (water potential equals turgor plus osmotic potential) [21], [32]. Analyzing these properties significantly contribute to our understanding of growth or stress responses and adaptations at the cellular level.

In addition to its original function of probing of cell turgor, the pressure probe facilitated sampling and appeared to be a critical instrument for integrative studies; vibration-free

manipulation of the microcapillary, and online monitoring of the operation by a stereomicroscope were quite necessary for precise sampling from a predetermined location. On the other hands, a pressure probe sensitive pressure transducer connected to a digital gauge provided observing the pressure change inside the microcapillary when the tip entered in the plant. Furthermore, a pressure probe provides negative pressure which is reasonably necessary to extract sufficient sap when cells have insufficiently low turgor pressure and positive pressure to push the sap out of the microcapillary glass after sampling of cell sap. The cell pressure probe could be successfully used for accessing parenchyma cells.

Several metabolites from mono- to oligosaccharides, amino acids, secondary metabolites and organic acids could be detected with both soft ionization mass spectrometry techniques examined here (Fig. 3 and Table 1). NanoESI-MS, however, yielded more metabolite signals in both negative and positive modes. MALDI-MS with our selected matrices could yield metabolite signals only in positive ion mode. Thus, we concluded that nanoESI MS is the preferred technique for profiling negatively charged metabolites. Finding a proper matrix for a specific group of chemicals is a big challenge in UV-MALDI-MS since there are no clear criteria for the selection of the matrix and it is mostly empirical [18]. In the shotgun metabolite profiling, a wide range of compounds from carbohydrates, amino acids, organic acids, secondary metabolites and fatty acids are examined. Even after sample purification and separation, diverse types of metabolites may still exist in the mixture.

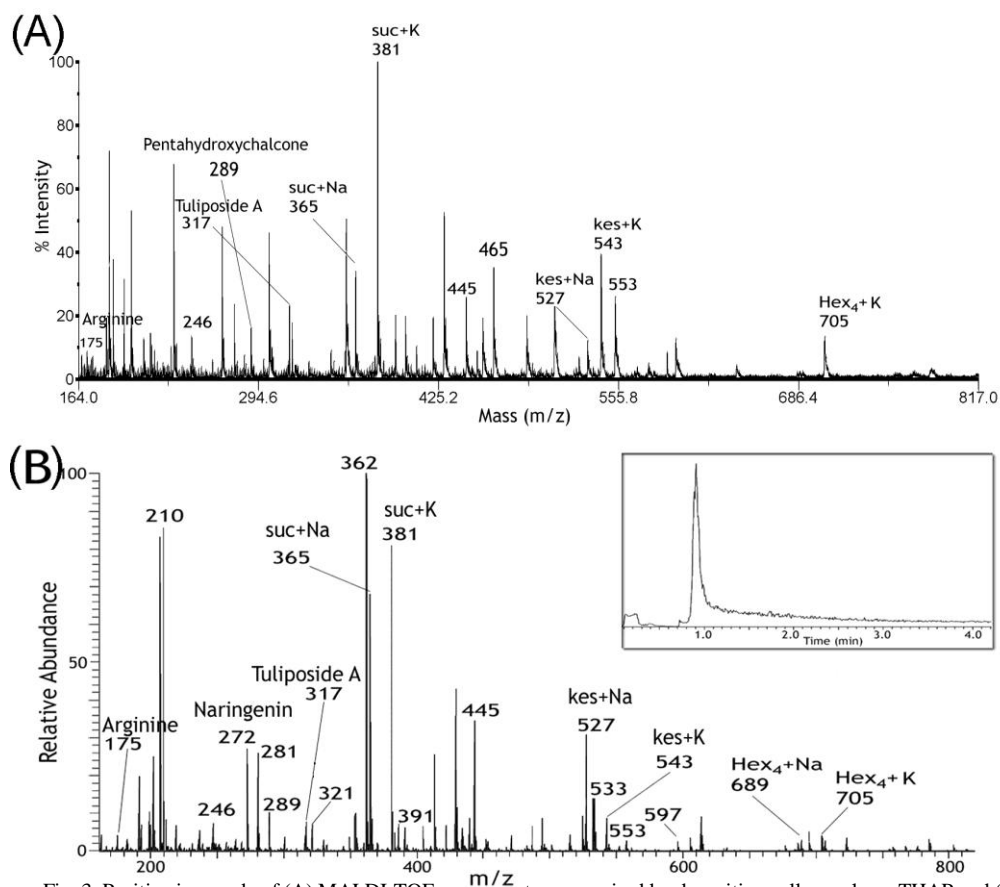


Fig. 3. Positive ion mode of (A) MALDI-TOF mass spectrum acquired by depositing cell sample on THAP and (B) nanoESI-Orbitrap mass spectrum acquired by injecting picoliter cell sample from a tulip bulb parenchyma cell. Inset in (B) shows chromatogram of cell sample mass spectrum. For a detail list of detected metabolites see Table 1.

TABLE 1

METABOLITES IDENTIFIED IN TULIP BULB SINGLE-CELL SAMPLES BY NANOESI-MS. THOSE WITH ASTERISK WERE ALSO DETECTED BY MALDI-MS.

m/z of Detected signal	interpretation	ion	Exact m/z	Δm (ppm)
88.0390	alanine	[M-H]-	88.0393	3.4
90.0557	alanine *	[M+H]+	90.0549	-8.9
99.0445	tulipalin A	[M+H]+	99.0440	-5.0
104.0712	GABA *	[M+H]+	104.0706	-5.8
104.1075	choline	[M+H]+	104.1070	-4.8
115.0024	maleic acid	[M-H]-	115.0026	1.7
115.0370	tulipalin B	[M+H]+	115.0371	0.9
115.0371	glycerol	[M+Na]+	115.0366	-4.3
122.0232	nicotinate	[M-H]-	122.0238	4.9
128.0112	alanine *	[M+K]+	128.0108	-3.1
133.0132	malic acid	[M-H]-	133.0132	0.0
133.0607	asparagine	[M+H]+	133.0608	0.8
146.0449	glutamic acid	[M-H]-	146.0449	0.0
148.0594	glutamic acid *	[M+H]+	148.0604	6.8
154.0255	proline *	[M+K]+	154.0263	5.2
156.0764	histidine	[M+H]+	156.0768	2.6
167.0336	coumaric acid	[M-H]-	167.0339	1.8
171.0155	asparagine	[M+K]+	171.0166	6.4
175.0245	ascorbic acid	[M-H]-	175.0237	-4.6
175.1189	arginine *	[M+H]+	175.1178	-6.3
185.0308	glutamine	[M+K]+	185.0323	8.1
191.0193	citric acid	[M-H]-	191.0185	-4.2
195.0502	gluconic acid	[M-H]-	195.0504	1.0
203.0512	hexose	[M+Na]+	203.0526	6.9
219.0251	hexose *	[M+K]+	219.0265	6.4
226.0712	arogenic acid	[M-H]-	226.0710	-0.9
272.0688	naringenin chalcone	[M+H]+	272.0685	-1.1
288.0630	pentahydroxychalcone*	[M+H]+	288.0634	1.4
317.0609	tuliposide A *	[M+K]+	317.0633	7.6
341.1089	sucrose	[M-H]-	341.1077	3.3
365.1025	sucrose *	[M+Na]+	365.1053	7.7
381.0763	sucrose *	[M+K]+	381.0792	7.6
503.1588	kestose	[M-H]-	503.1607	3.3
527.1611	kestose *	[M+Na]+	527.1583	-5.3
543.1304	kestose *	[M+K]+	543.1322	3.3
665.2125	nystose	[M-H]-	665.2135	3.3
689.2141	nystose *	[M+Na]+	689.2111	-4.4
705.1821	nystose *	[M+K]+	705.1850	4.1
827.2682	fructosylnystose	[M-H]-	827.2663	3.3
867.2349	fructosylnystose *	[M+K]+	867.2378	3.3

For UV-MALDI MS metabolite profiling of plant cell samples, THAP and DHB have been commonly used among organic matrixes. In the case of THAP, with an almost uniform deposition on the plate, the possibility of the co-existence of sample and matrix molecules in a location on the plate is quite high, compared to the DHB with an extremely localized crystallization [22]. Additionally, more diverse metabolites can be detected with THAP. We have introduced a number of new matrixes for UV-MALDI MS metabolite profiling of plants including the nanoparticles (NPs) of titanium silicon oxide ((SiO₂)(TiO₂), barium strontium titanium oxide ((BaTiO₃)(SrTiO₃), titanium oxide (TiO₂) [18] and carbon nanotubes (CNTs) [33]. For this study, we used THAP, due to its high signal acquisition yield, and TiO₂ NPs, due to their applicability to quantitative analyses.

Sugars including simple saccharides and fructans play important roles in plant cell growth and stress tolerance. Nanoparticles are powerful matrixes for UV-MALDI MS

analyses of underivatized carbohydrates [18]. High linearity response (Fig. 4) and low limit of detection (LOD, Table 2) of NPs make them a choice for detecting and the quantifying underivatized carbohydrates UV-MALDI MS. Overall, MALDI-MS seemed to be superior technique for quantitation of underivatized sugars in this work (Table 2). Carbohydrates soluble in cell sap are in an aqueous solution containing cations such as K⁺, Na⁺, as well as Mg²⁺ and Ca²⁺. Therefore, soluble carbohydrates are detected in positive ion mode mainly as potassiumated species (M + K)⁺ and in negative mode as deprotonated species ([M-H]⁻) [18], [33]–[35].

A challenge in the shotgun metabolite profiling by nanoESI-MS is the signal suppression. As it is known in ESI MS the suppression can be originated from abundant salts which naturally exist in cell solutions, interfering metabolites such as lipids, and from the competition between biomolecules during the ionization [22]. The advantage of UV-MALDI MS is its robustness under high salt concentration. We have frequently observed a higher relative signal abundance of many metabolites in MALDI mass spectra compared to their abundance in ESI mass spectra [22]. Overall, our experience indicates that single-cell MALDI MS is more efficient for the analysis of underivatized, plant-derived carbohydrates [22].

In addition to signal suppression, the lower signal intensity of plant metabolites when analyzing picoliter cell sap samples by using ESI-Orbitrap MS may be resulted by peak broadening in chromatograms. The peak broadening may result in the loss of signal of less abundant metabolites. Unlike MALDI-MS, in ESI-MS sample is not analyzed as a package and shortening of analysis time should be taken into account. For optimizing the analysis, we applied a mixture of spraying solution (aqueous MeOH 50%), an increased flow rate, and reducing the distance between injection location and ion source. Application of nanoESI instead of a microESI ion source provided higher salt resistance for the ionization and also less peak broadening (Fig. 3B).

TABLE 2
LOD AND DYNAMIC RANGE OF STANDARD SUCROSE

	LOD (pmol)	Dynamic range of detection (pmol)	range of linearity signal intensity vs. pmol of sucrose
MALDI-TOF	2	2-25000	5-640
nanoESI-Orbitrap	5	5-2500	10-320

By using data of cell sap sample volume (measured after single-cell sampling) and the number of mole of analyte in each cell sample (measured by using nanoESI and MALDI mass spectrometry techniques) sucrose and kestose abundance was quantified in several cells at different locations of same scale tissue (Fig. 5A and 5B). The quantitative profiles generated by these two techniques looked similar and the average of concentration of sucrose and kestose in the population of examined cells measured by two techniques was not significantly different (Fig. 5C). In summary, storage parenchyma cells of the bulb showed similarity in their sucrose and kestose content. The variation

in the abundance of sucrose and kestose observed in the metabolite profiles can be attributed to the natural cell-to-cell variation.

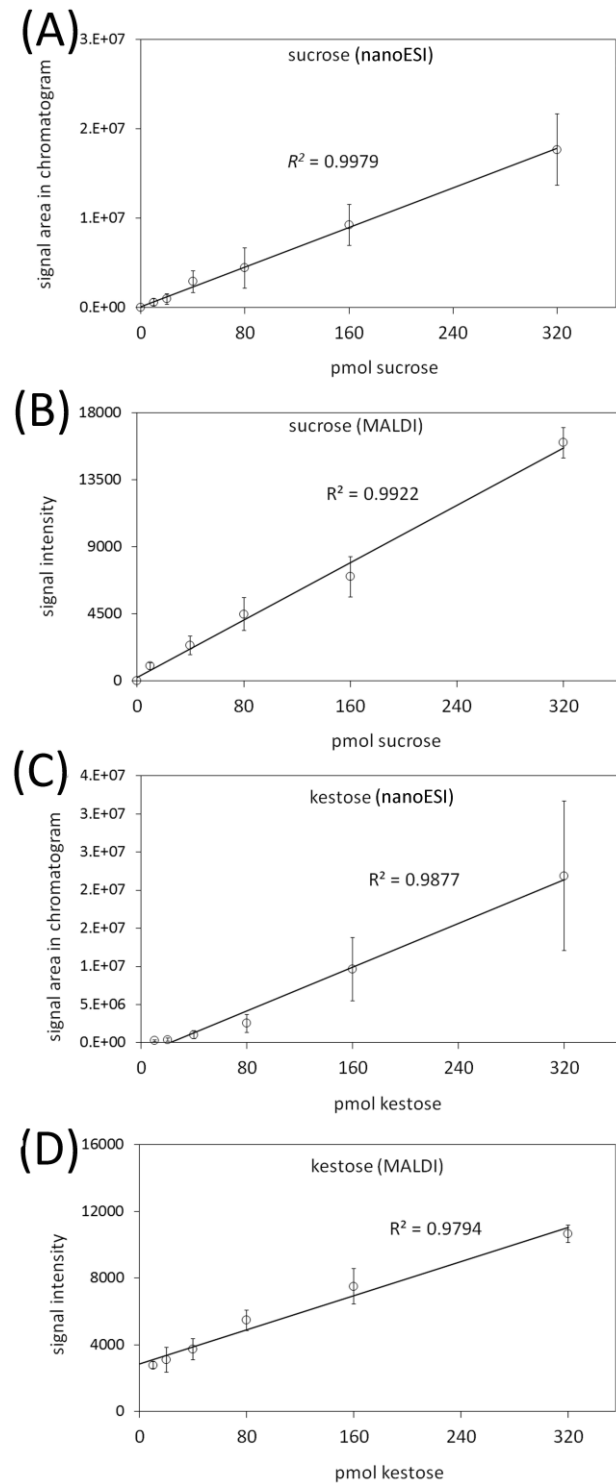


Fig. 4. Linear relationship of signal intensity vs. pmol of sucrose (A and B) and kestose (C and D) examined by using ESI-MS (A and C) and MALDI-MS (B and D).

After co-plotting turgor values and sucrose concentration of parenchyma cells, an interesting pattern appeared (Fig. 6). Sucrose is the dominant metabolite in starch-storing parenchyma cells of cooled tulip bulbs [24], [25]. Consequently, it can be expected the osmotic potential of

those cells would be strongly influenced by the concentration of sucrose. Assuming that cells with similar morphology and physiological phenotype in a specific location of a plant tissue would have similar water potential, turgor must be accordingly influenced directly by the concentration of sucrose in those parenchyma cells.

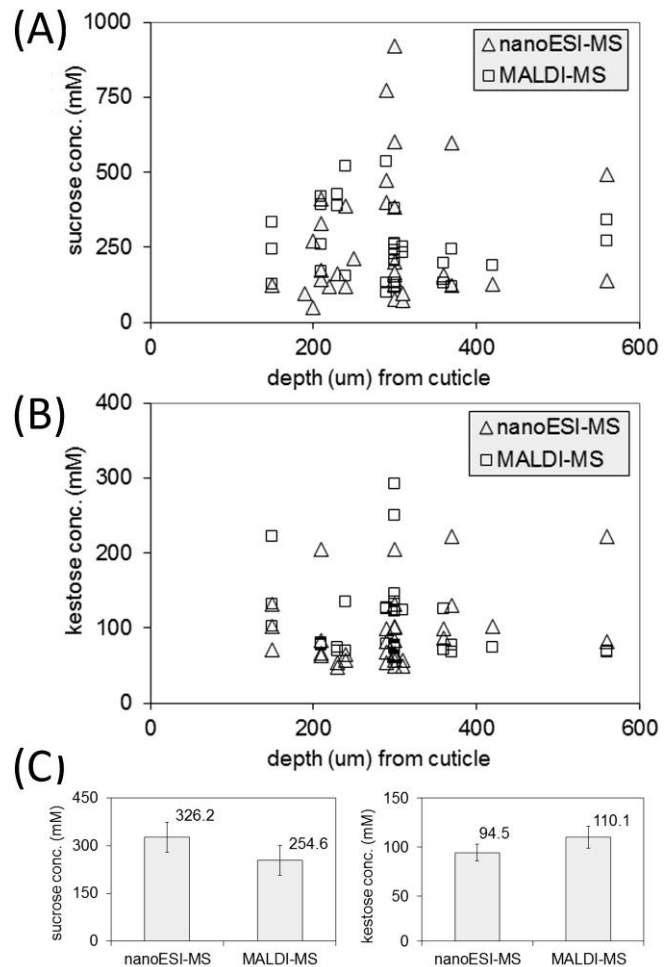


Fig. 5. Abundance of sucrose (A) and kestose (B) in parenchyma cells located at different part of the second scale of tulip bulb as measured by using nanoESI-MS and MALDI-MS. (C) Graphs show average concentrations of sucrose and kestose extracted from data at (A) and (B).

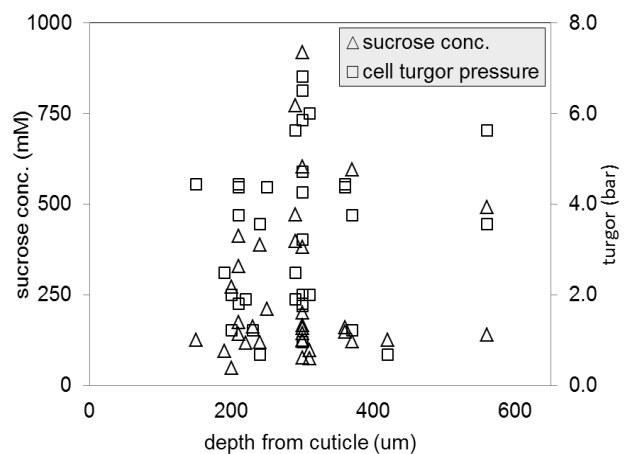


Fig. 6. Sucrose concentration in parenchyma cells measured by nanoESI-MS is co-plotted with the turgor pressure of corresponding cells.

IV. CONCLUSION

The joint application of a pressure probe and a mass spectrometer system facilitates the acquisition of data about the water status and the molecular composition of *in situ* living single cells. Measuring of turgor by a pressure probe and sampling of single-cell sap followed by mass spectrometry metabolite profiling showed adaptability for fast analysis of physiological status of intact plant cells. The result showed plant growth or responses to environmental stresses can be monitored with molecular precision and a single-cell resolution level and therefore wider insights to the cellular events can be achieved. This integrative analysis provides a “speaking plant cell approach” by which crop growth and agro-chemical application especially for the production under structure can be optimized. Although underivatized sugars are difficult to ionize, utilization of nanoESI-MS and MALDI-MS with selected matrices resulted in efficient characterization and quantitation of major soluble sugars in plant cells. Single-cell metabolomics is the analysis of the phenotype with the highest resolution and has great potential to contribute the enhancement of cell systems biology. The shotgun approach is very beneficial to metabolomics since reduced steps are included in workflow of the analysis.

REFERENCES

- [1] Kehr, J. Single cell technology. *Curr. Opin. Plant Biol.* 6, 617-621 (2003).
- [2] G. Angeles, J. Berrio-Sierra, J. P. Joseleau, P. Lorimier, A. Lefebvre, K. Ruel, Preparative laser capture micro-dissection and single pot cell wall material preparation: a novel method for tissue specific analysis. *Planta*, 224, 228-232, 2006.
- [3] D. Holscher, B. Schneider, Laser micro-dissection and cryogenic nuclear magnetic resonance spectroscopy: an alliance for cell type-specific metabolite profiling. *Planta*, 225, 763-770, 2007.
- [4] S. H. Li, B. Schneider, J. Gershenzon, Microchemical analysis of laser-microdissected stone cells of Norway spruce by cryogenic nuclear magnetic resonance spectroscopy. *Planta*, 225, 771-779, 2007.
- [5] B. Shrestha, A. Vertes, In situ metabolic profiling of single cells by laser ablation electrospray ionization mass spectrometry. *Anal. Chem.*, 81, 8265-8271, 2009.
- [6] 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, 2304-2311, 2009.
- [7] D. Hüskén, E. Steudle, U. Zimmermann, Pressure probe technique for measuring water relations of cells in higher plants. *Plant Physiol.*, 61, 158-163, 1978.
- [8] 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, 1841-1848, 2008.
- [9] M. Malone, R. A. Leigh, A. D. Tomos, Extraction and analysis of sap from individual wheat leaf cells: the effect of sampling speed on the osmotic pressure of extracted sap. *Plant Cell Environ.*, 12, 919-926, 1987.
- [10] M. Malone, R. A. Leigh, A. D. Tomos, Concentration of Vacuolar Inorganic Ions in Individual Cells Of Intact Wheat Leaf Epidermis. *J. Exp. Bot.*, 42, 305-309, 1991.
- [11] O. A. Koroleva, J. F. Farrar, A. D. Tomos, C. J. Pollock, Patterns of solute in individual mesophyll, bundle sheath and epidermal cells of barley leaves induced to accumulate carbohydrate. *New Phytol.*, 136, 97-104, 1997.
- [12] O. A. Koroleva, J. F. Farrar, A. D. Tomos, C. J. Pollock, Carbohydrates in individual cells of epidermis, mesophyll, and bundle sheath in barley leaves with changed export or photosynthetic. *Plant Physiol.*, 118, 1525-1532, 1998.
- [13] A. V. Korolev, A. D. Tomos, R. Bowtell, J. F. Farrar, Spatial and temporal distribution of solutes in the developing carrot taproot measured at single-cell resolution. *J. Exp. Bot.*, 51, 567-577, 2000.
- [14] A. D. Tomos, R. A. Sharrock, Cell sampling and analysis (SiCSA): metabolites measured at single cell resolution. *J. Exp. Bot.*, 52, 623-630, 2001.
- [15] S. P. Markey, Quantitative mass spectrometry. *Biol. Mass Spectrom.*, 8, 426-430, 1981.
- [16] D. J. Harvey, Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides. *Rapid Commun. in Mass Spectrom.*, 7, 614-619, 1993.
- [17] M. Bantscheff, M. Schirle, G. Sweetman, J. Rick, J. Bernhard, B. Kuster, Quantitative mass spectrometry in proteomics: a critical review *Anal. Bioanal. Chem.*, 389, 1618-2650, 2007.
- [18] Y. Gholipour, H. Nonami, R. Erra-Balsells, Diamond, titanium dioxide, titanium silicon oxide and barium strontium titanium oxide nanoparticles as matrices for direct matrix-assisted laser desorption/ionization mass spectrometry analysis of carbohydrates in plant tissue, *Anal. Chem.*, 82, 5518-5526, 2010.
- [19] P.J. Kramer, and J.S. Boyer, *Water Relations of Plants and Soils*, Academic Press, San Diego, 1995.
- [20] J.S. Boyer, *Measuring the Water Status of Plants and Soils*, Academic Press, San Diego, 1995.
- [21] H. Nonami, *Plant Water Relations* (Japanese in text), Yokendo, Tokyo, 2001.
- [22] 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, DOI: 10.5702/massspectrometry. A0003, 2012.
- [23] 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 in Systems Biology and Bioengineering: Proceedings of The World Congress on Engineering 2012, WCE 2012, 4-6 July, 2012, London, U.K., pp. 572-577.
- [24] R. Moe, A. Wickström A, The effect of storage temperature on shoot growth, flowering, and carbohydrate metabolism in tulip bulbs, *Physiol Plant*, 28, 81-87, 1973.
- [25] H. Lambrechts, F. Rook, C. Kolloff C, Carbohydrate status of tulip bulbs during cold-induced flower stalk elongation and flowering. *Plant Physiol*, 104, 515-520, 1994.
- [26] E. Steudle, U. Zimmermann, Effect of turgor pressure and cell size on the wall elasticity of plant cells, *Plant Physiol.*, 59, 285, 1977.
- [27] P. B. Green, R. O. Erickson, and J. Buggy, Metabolic and physical control of cell elongation rate: in vivo studies in nitella., *Plant Physiol.*, 47, 423, 1971.
- [28] H. Nonami, and J.S. Boyer, Wall extensibility and cell hydraulic conductivity decrease in enlarging stem tissues at low water potentials., *Plant Physiol.*, 93, 1610-1619, 1990.
- [29] H. Nonami, and J.S. Boyer, Direct demonstration of a growth-induced water potential gradient. *Plant Physiol.*, 102, 13-19, 1993.
- [30] M. Malone, and A.D. Tomos, A simple pressure-probe method for the determination of volume in higher-plant cells, *Planta*, 182, 199-203, 1990.
- [31] K. A. Shackel, Direct measurement of turgor and osmotic potential in individual epidermal cells: independent confirmation of leaf water potential as determined by in situ psychrometry., *Plant Physiol.*, 83, 719-722, 1987.
- [32] H. Nonami, and 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 nanoliter osmometer, *Planta*, 171, 35-46, 1989.
- [33] Y. Gholipour, H. Nonami, R. Erra-Balsells, In situ analysis of plant tissue underivatized carbohydrates and on-probe enzymatic degraded starch by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by using carbon nanotubes as matrix, *Analytical Biochemistry*, vol. 383, pp. 159-167, 2008.
- [34] B. Stahl, A. Linos, M. Karas, F. Hillenkamp, M. Steup, Analysis of fructans from higher plants by matrix-assisted laser desorption/ionization mass spectrometry, *Anal. Biochem.* 246, 195-204, 1997.
- [35] D.J. Harvey, Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: an update covering the period 2001-2002, *Mass Spectrom. Rev.* 27, 125-201, 2008.