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In Situ Molecular Analysis of Plant Tissues by Live Single-Cell Mass Spectrometry

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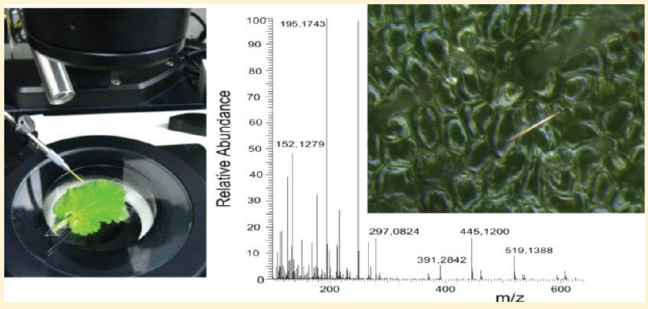
Supporting Information

ABSTRACT: We report the development of a rapid, direct molecular analysis of live, single plant cells viewed under a video microscope in their natural environment. A nanoelectrospray tip was used to extract the contents of a single leaf, stem, or petal cell from *Pelargonium zonale*, and the samples were analyzed on an Orbitrap mass spectrometer by nanoelectrospray ionization. Around a thousand *m*/*z* peaks belonging to metabolites and other compounds in each sample were obtained and processed by using statistical tools to find the cell specific molecular peaks. Hybrid high-resolution mass spectrometry analysis was performed to confirm the structure of specific metabolites from the analyzed samples. This method is useful for identifying specific molecules in live single cells from plant tissue and will allow different cell types and stages from different sites in the plant to be compared with morphological observations.



The aim of plant biochemistry is to fully understand the molecular mechanisms taking place in each part of the plant and then to control those processes. Plant systems can be studied by examining which genes are being expressed[1] and which proteins are present;[2−4] however, our interests lie in discovering, for instance, what causes certain plants to produce more terpenoids than others under the same growing conditions.[5]

The dynamic functioning of plant cells produces small-molecule products known as metabolites, which are important for determining which proteins or enzymes have been activated. Profiling the metabolites of plants is thus very important for understanding how plants function[1,6,7] and for distinguishing plant genotypes.[8]

Plant metabolites are extremely diverse, and many of these compounds are easily oxidized. Therefore, the extraction process and the equipment used are key factors in the analytical protocol. Bulk plant extracts have previously been the main resource in plant biochemistry, although this provides information on a mixture of all the tissues present in the sample. Current analytical methods, such as nuclear magnetic resonance (NMR), Fourier transform-infrared spectroscopy, and mass spectrometry (MS) are often combined with chromatography[9,10] that requires samples of several microliters in volume, which are not always easy to obtain. Ambient ionization MS techniques,[11] such as desorption electrospray ionization,[12] have been developed and allow the direct analysis of a surface. Direct analyses of tissues by methods such as tissue spray ionization[13] and leaf spray ionization[14] are also developed recently.

Life is however dynamic on a microscale in localized cells, which perform specific tasks in various tissues. Accordingly, it is important to develop dynamic, direct, and exhaustive molecular analysis of individual cells under microscopic morphological observation[15] and to establish live single-cell MS.[16,17]

Several studies have attempted to improve the methods for analyzing the chemical composition of a group of cells in the same tissue[18−20] or of even single cells.[21−24] Single-cell analysis in plant tissues is different from that of cultured cells, because of the plant tissues' irregular surfaces, higher dilution of biomolecules in the cell, and strong cellulose walls. These are factors to be considered when designing a method for direct single-cell analysis in undamaged plant tissue.

MS has been applied to metabolic analysis at the single-cell level.[23,24] High-resolution mass spectrometers, such as a hybrid linear ion trap/Orbitrap mass spectrometer, have enabled the identification of both common and uncommon compounds.[25,26] The combination of video-microscopic imaging and MS has been used to directly perform live single-cell analyses.[16,17,27,28] We have extended this technique for directly characterizing live plant tissues to provide fast, versatile, and nondestructive analysis of different single cells in several types of plant tissues. This allows the direct collection of information

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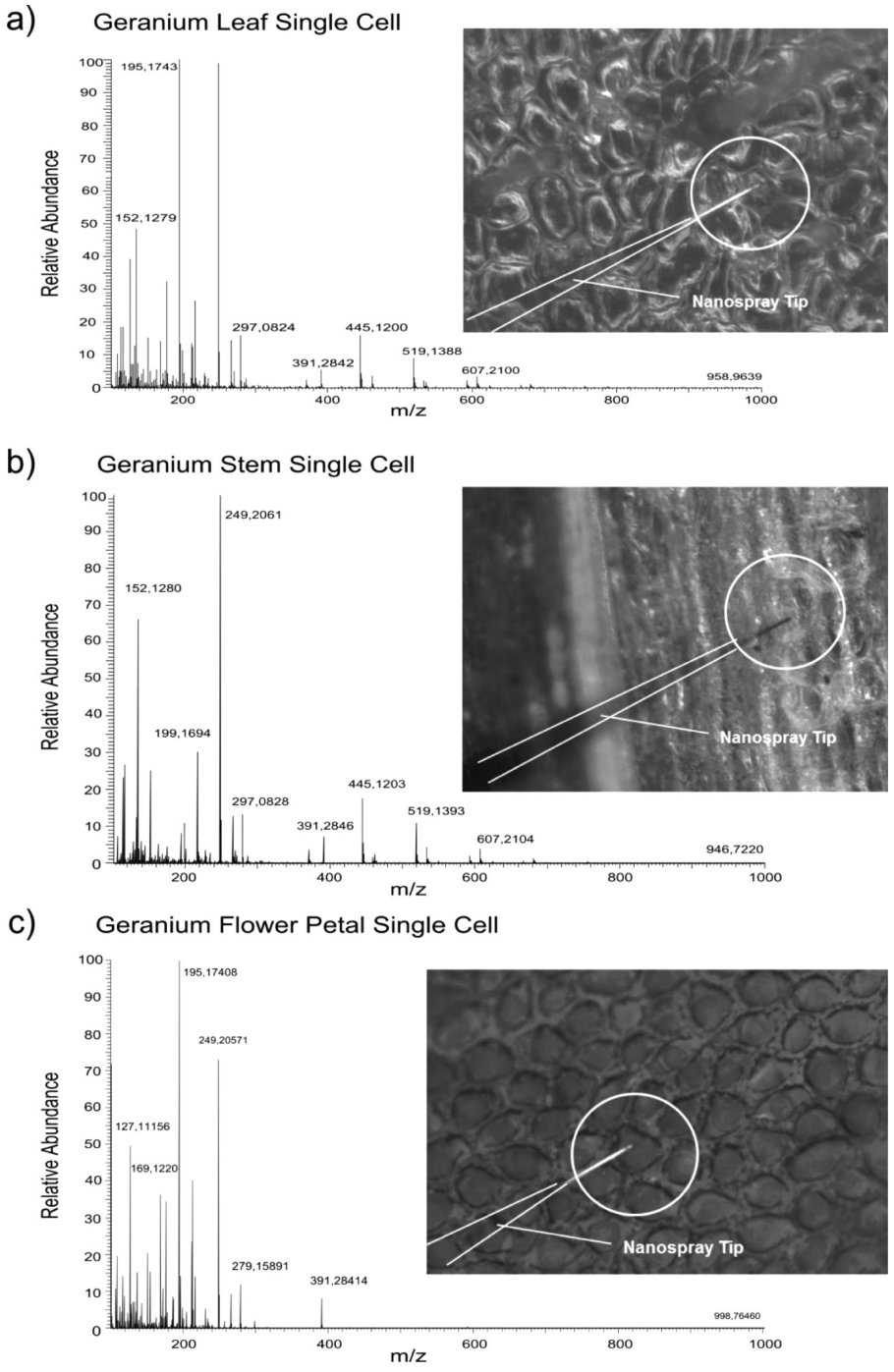

Figure 1. Example of full spectra (range *m/z*, 100−1000) and photographs of *Pelargonium zonale* single-cell contents extraction by the nanoelectrospray tip for the (a) leaf cell, (b) stem cell, and (c) flower petal cell.

at the cellular level in real time. If the analysis does not damage the plant tissue, metabolic processes can be monitored in situ together with morphological changes in various plant tissues.

In our previous study,[29] direct analysis of single plant cells was performed and molecules were successfully detected in stem and leaf cells. Herein, we present an overview of the performance of this single-cell analysis of live, undamaged plant tissues. Considering the morphology and characteristics of plant cells, we evaluate the usefulness and effectiveness of this approach for studying plant metabolite dynamics and indentifying new molecules.

EXPERIMENTAL SECTION

Plant Material. *Geraniaceae Pelargonium zonale* (L.) L'Hérit, known as the garden geranium, was used for this study. Leaves and stems were cut from healthy plants, and petals were taken from undamaged cut flowers that had bloomed for at least 2 days after opening. All tissues were analyzed within 5 min of collection and superficially cleaned with distilled water to remove any dust or dirt particles. Direct contact with the tissue was avoided in order to prevent damage and alteration of the microstructure. The specimens were mounted over a thin glass strip and kept still at the edges.

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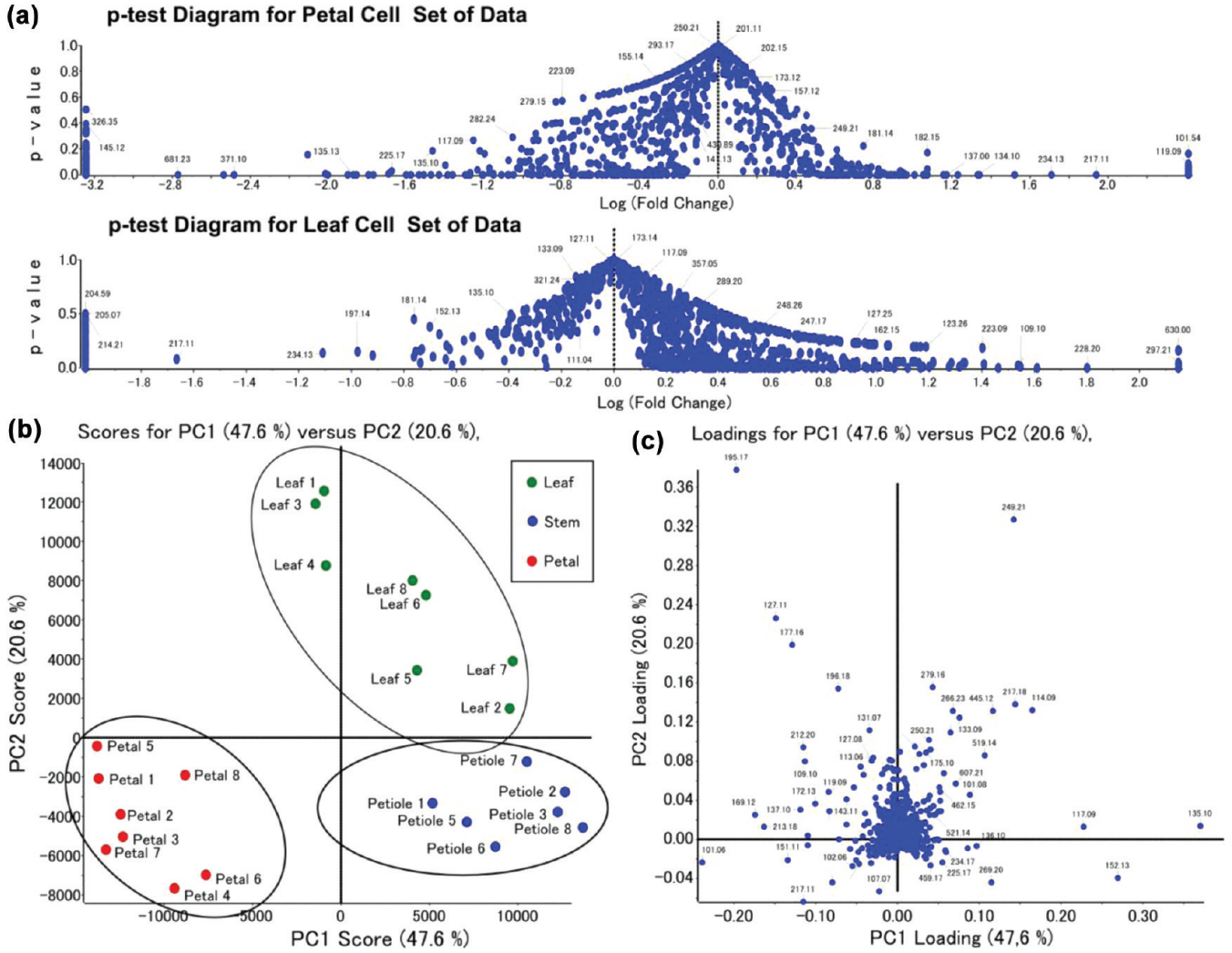

Figure 2. (a) Statistical analysis of *Pelargonium zonale* data: *p*-value diagrams of log fold change vs *p*-value for the petal peaks and for leaf specific peaks. (b) PCA applied to peak intensities of direct single cell analysis data of the leaf set, petal set, and stem set of samples. Each group shows clusters in two-dimensional space provided by two vectors of principal components (PC1 = 47.6% and PC2 = 20.6%). (c) PC1 and PC2 loadings applied to all m/z peaks detected in the leaf, stem, and petal sample spectra. The further the m/z peak is from the origin in the PC1 and PC2 coordinates, the more specificity it is thought to have by statistic calculations.

General Experimental Procedures and Materials. Specimens of leaf, stem, and petal tissues were monitored with a charge-coupled device (CCD) video camera mounted on a stereomicroscope (Olympus SZX12, Japan or Leica DFC 400, Germany). By using a syringe, cell contents were sucked via tubing into a gold-coated glass capillary nanoelectrospray tip (1 μm top diameter, HUMANIX, Japan) set on a micromanipulator (Narishige, MHW-103, Japan) (videos in the Supporting Information). For positive ion mode detection, LC−MS grade acetonitrile (0.3 μL) containing formic acid (0.5%, 99% purity) was added from a micropipet to the sample in the capillary nanoelectrospray tip immediately before analysis. The choice of solvent for general analysis was based on data collected about different solvents and mixtures in order to ensure good results and reproducibility.[30,31] Acetonitrile containing formic acid (0.5%, 99% purity) improved the definition of the spectra. Therefore, this mixture was used as a general solvent for the samples.

Standards used for confirmation of structures were bought from Sigma-Aldrich and TCI Laboratory Chemicals (Japan), and racemic mixture standards with concentrations of 0.01 mM and 1 μM were prepared for the analysis.

Mass Spectrometry. MS analysis was performed on an apparatus (LTQ Orbitrap XL, Thermo Fisher Scientific Inc.) equipped with a nanoelectrospray ion source. The spray voltage was set between 1500 and 2000 V, with a distance of 3−5 mm between the top of the needle and the of mass spectrometer inlet. The resolution of the equipment was set at 100 000, and the capillary temperature was set at 50 °C. The selected range of measurement was from m/z 100 to 1000 . For MS/MS analysis, the high-energy collision-induced dissociation (HCD) mode was selected at a normalized collision energy from 55% to 85% depending on the compound being analyzed. For MS^3 analysis, the collision-induced dissociation (CID) mode was used for selected peaks using 25% to 35% normalized collision energy and an activation (q) of 0.25. The orbitrap automated gain control targets were set to 1×10^6 for a full scan and 1×10^5 for an MS^n scan. The electron multiplier gain, Fourier transform, storage transmission, and mass accuracy (FT) were manually calibrated immediately before measurements in the positive-mode using polytyrosine (m/z 182.081 17, m/z

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Table 1. Summarized Statistical Results for Single-Cell MS Data[a]

possible compounds	m/z $[M + H]^+$	t-value	p-value	Student's t test: leaf−stem	Student's t test: leaf−petal	Student's t test: stem−petal
(±)-(R, S)-limonene, α,β-pinene, myrcene, or comphene	137.132 40	1.419 35	0.179 33	−31.67	99.91	99.97
(−)-(R)-carvone/(−)-isopiperitenone	151.111 68	1.088 60	0.296 10	96.11	99.78	−99.97
geranial, (−)-*trans*-isopiperitenol/(±)-*trans*- carveol, perillyl alcohol, or (+)- pulegone/(−)-*cis*- isopulegone	153.127 36	1.748 99	0.165 70	82.08	99.23	97.32
geraniol, citronellal, α- terpineol, 1,8-cineole, sabinene hydrate, (−)-endofenchol, or (−)- menthone/(+)-isomenthone	155.142 94	1.032 33	0.159 62	86.90	98.99	99.47
citronellol or (−)- menthol/(+)-neomenthol	157.158 65	1.394 99	0.132 39	99.90	65.04	−99.63
geranic acid	169.122 23	0.513 20	0.616 42	97.29	−98.80	−99.98
methyl citronellate	185.153 54	1.589 69	0.182 44	75.95	99.36	97.56
geranyl acetate	197.153 41	1.084 28	0.297 94	24.54	97.22	65.11
blumenol B	227.164 11	0.380 31	0.709 85	99.19	25.39	−76.03

[a]Measured $[M + H]^+$ m/z peak, t-value, p-value, and t-test comparison for leaf versus stem, leaf versus petal, and stem versus petal.

Table 2. Nanoelectrospray Ionization MS Data[a]

compound	m/z $[M + H]^+$	theoretical mass	Δ (ppm)	RDB equiv	composition $[M + H]$	HCD tandem MS/MS product ions m/z
(±)-(R, S)-limonene	137.13240	137.13248	−0.56	2.5	$C_{10}H_{17}$	119 (C_9H_{11}), 95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7), 77 (C_6H_5)
α, β-pinene	″	″	″	″	″	95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7), 77 (C_6H_5)
myrcene	″	″	″	″	″	119 (C_9H_{11}), 95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7), 67 (C_5H_7)
comphene	″	″	″	″	″	95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7), 77 (C_6H_5)
(−)-(R)-carvone/ (−)-sopiperitenone	151.11168	151.11174	−0.41	3.5	$C_{10}H_{15}O$	133 ($C_{10}H_{15}O$), 123 ($C_8H_{11}O$), 109 (C_7H_9O), 107 (C_8H_{11}), 105 (C8H_9), 95 (C_8H_9), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7)
geranial	153.12736	153.12739	−0.21	2.5	$C_{10}H_{17}O$	135 ($C_9H_{11}O$), 109 (C_7H_9O), 107 (C_8H_{11}), 97 (C_6H_9O), 95 (C_6H_7O), 93 (C_6H_5O), 83 (C_5H_7O), 81 (C_6H_9), 79 (C_6H_7)
(−)-*trans*- isopiperitenol/ (±)-*trans*- carveol	″	″	″	″	″	135 ($C_9H_{11}O$), 109 (C_7H_9O), 107 (C_8H_{11}), 97 (C_6H_9O), 95 (C_6H_7O), 93 (C_7H_9), 81 (C_6H_9), 79 (C_6H_7)
perillyl alcohol	″	″	″	″	″	135 ($C_9H_{11}O$), 109 (C_7H_9O), 107 (C_8H_{11}), 81 (C_6H_9)
(+)- pulegone/ (−)-*cis*- isopilegone	″	″	″	″	″	135 ($C_9H_{11}O$), 109 (C_7H_9O), 107 (C_8H_{11}), 97 (C_6H_9O), 95 (C_6H_7O), 81 (C_6H_9), 79 (C_6H_7)
geraniol	155.14295	155.14304	−0.66	1.5	$C_{10}H_{19}O$	137 ($C_{10}H_{17}$), 111 ($C_7H_{11}O$), 109 (C_8H_{13}), 98 (C_7H_{15}), 97 (C6H_9O), 95 (C_6H_7O), 93 (C_7H_9), 81 (C_6H_9), 67 (C_5H_7).
citronellal	155.14295	155.14304	−0.66	1.5	$C_{10}H_{19}O$	137 ($C_9H_{13}O$), 127 ($C_8H_{15}O$), 113 ($C_7H_{13}O$), 111 ($C_7H_{11}O$), 109 (C_7H_9O), 99 ($C_6H_{11}O$), 97 (C_6H_9O), 83 (C6H_{11}), 81 (C_6H_9)
α-terpineol	″	″	″	″	″	137 ($C_{10}H_{17}$), 111 ($C_7H_{11}O$), 109 (C_8H_{13}), 98 ($C_6H_{11}O$), 97 (C_6H_9O), 95 (C_7H_{11}), 93 (C_7H_9)
1,8-cineole	155.14295	155.14304	−0.66	1.5	$C_{10}H_{19}O$	137 ($C_9H_{13}O$), 127 ($C_8H_{15}O$), 113 ($C_7H_{13}O$), 111 ($C_7H_{11}O$), 109 (C_7H_9O), 99 ($C_6H_{11}O$), 83 (C_6H_{11}), 81 (C_6H_9)
sabinene hydrate	″	″	″	″	″	137 ($C_9H_{13}O$), 113 ($C_7H_{13}O$), 111 ($C_7H_{11}O$), 109 (C_7H_9O), 99 ($C_6H_{11}O$), 97 (C_6H_9O), 83 (C_6H_{11}), 81 (C6H_9)
(−)-endofenchol	″	″	″	″	″	137 ($C_9H_{13}O$), 113 ($C_7H_{13}O$), 111 ($C_7H_{11}O$), 109 (C_7H_9O), 99 ($C_6H_{11}O$), 83 (C_6H_{11}), 81 (C_6H_9)
(−)- menthone/ (+)-isomenthone	″	″	″	″	″	137 ($C_9H_{13}O$), 127 ($C_8H_{15}O$), 113 ($C_7H_{13}O$), 111 ($C_7H_{11}O$), 109 (C_7H_9O), 99 ($C_6H_{11}O$), 97 (C_6H_9O), 83 (C_6H_{11}), 81 (C_6H_9)
citronellol	157.15865	157.15869	−0.27	0.5	$C_{10}H_{21}O$	139 ($C_9H_{15}O$), 129 ($C_{10}H_9$), 121 (C_9H_{13}), 111 ($C_7H_{11}O$), 99 ($C_6H_{11}O$), 97 (C_6H_9O), 95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 83 (C_5H_7O), 81 (C_6H_9), 79 (C_6H_7), 71 (C_4H_7O), 69 (C_5H_9), 67 (C_5H_7), 55 (C_4H_7)
(−)- menthol/ (+)-neo menthol	″	″	″	″	″	139 ($C_9H_{15}O$), 121 (C_9H_{13}), 111 ($C_7H_{11}O$), 99 ($C_6H_{11}O$), 97 (C_6H_9O), 95 (C_7H_{11}), 93 (C_7H_9), 91 (C_7H_7), 81 (C_6H_9), 79 (C_6H_7)
geranic acid	169.12223	169.122231	−0.45	2.5	$C_{10}H_{17}O_2$	127 ($C_7H_{11}O_2$), 123 ($C_8H_{11}O$), 95 (C_6H_7O), 85 ($C_4H_5O_2$)
methyl citronellate	185.15354	185.15361	−0.36	1.5	$C_{11}H_{21}O_2$	157 ($C_9H_{17}O_2$), 143 ($C_9H_{16}O_2$), 141 ($C_8H_{13}O_2$), 129 ($C_7H_{13}O_2$)
geranyl acetate	197.15341	197.15361	−1.00	2.5	$C_{12}H_{21}O_2$	179 ($C_{11}H_{15}O_2$), 141 ($C_8H_{13}O_2$), 137 ($C_8H_9O_2$), 123 ($C_{18}H_{11}O$), 119 (C_8H_7O), 109 (C_7H_9O), 105 (C_7H_5O), 95 (C_7H_{11}), 93 (C_7H_9), 91(C_7H_7),81(C_6H_9)
blumenol B	227.16411	227.16411227	−0.27	2.5	$C_{13}H_{23}O_3$	196 ($C_{13}H_{24}O_3$), 185 ($C_{10}H_{17}O_3$), 171 ($C_{13}H_{15}$), 157 ($C_{12}H_{13}$), 155 ($C_9H_{15}O_2$), 143 ($C_{11}H_{11}$), 137 ($C_9H_{13}O$), 109 (C_8H_{13}), 111 ($C_7H_{11}O$), 107 (C_7H_7O), 97 (C_6H_9O), 95 (C_7H_{11}), 93 (C_7H_9), 81 (C_6H_9)

[a]Ditto mark (″) indicates same value as cell above.

508.207 83, m/z 997.397 810) according to the manufacturer's instructions. The spectra were analyzed and evaluated using Xcalibur 2.0 (Thermo Fisher Scientific Inc.).

Data Processing. Data were processed by using Marker View 1.1 and 1.2 (AB Sciex). After normalization, the peak detection parameters were as follows: noise threshold, 1 count; minimum spectral width, 50 ppm; subtraction multiply factor, 1.3; and m/z tolerance, 50 ppm. Student's t test (t test) analysis of the data was performed for comparison between two sites in plant tissues, and principal component analysis (PCA) for all

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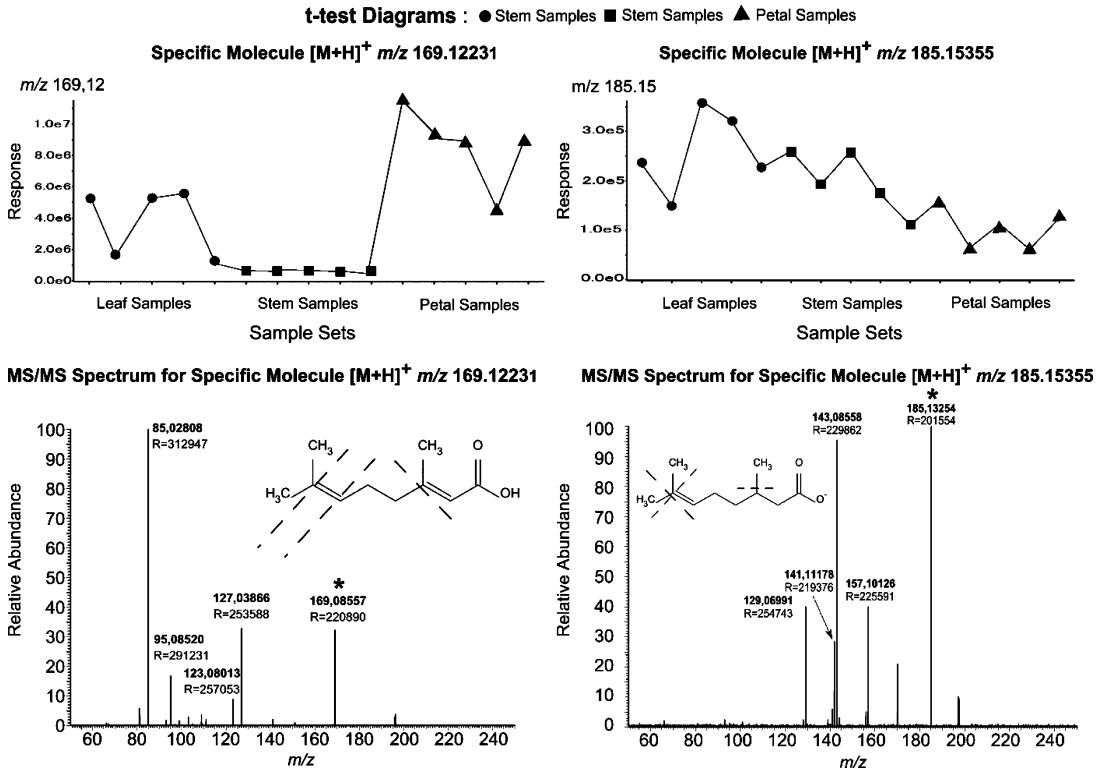

Figure 3. Specific molecule *t*-test diagram for peaks m/z 169.12231 $[M + H]^+$ and m/z 185.15355 $[M + H]^+$, identified as geranic acid and methyl citronellate, respectively. MS/MS spectra of parent peaks m/z 169.12231 $[M + H]^+$ (HCD 55%) and m/z 185.15355 $[M + H]^+$ (HCD 65%) for structural confirmation. See total composition in Table 2. *R*, peak resolution; ∗, parent ion.

data sets was performed for classifying the data. This PCA analysis is also used for first screening whether the analysis performed correctly. If clogging of the nanospray tip or no sucking of the cell contents occur, the data shows quite a dispersed point in the score plots. All processed samples were subjected to a *t* test and PCA for each specific m/z peak search.

Procedure for Live Plant Single-Cell Mass Spectrometry. A nanoelectrospray tip set on a micromanipulator was directly inserted into a single stem, leaf, or petal cell from *Pelargonium zonale* under the microscope, and the cell contents were removed. Because of the turgor pressure in plant cells, once the tip passed through the cellulose wall, a part of the cell contents was pushed into the tip. The removal of the cell contents was aided by a syringe connected to the tip with tubing. When the leaf cell contents were removed, particular care was taken to take the sample from a cell in the upper mesophyll, below the cuticle of the leaf (Figure 1a). A similar procedure for the removal of cell contents was used for the stem (Figure 1b) and petals (Figure 1c). The samples were analyzed using an Orbitrap mass spectrometer by nanoelectrospray ionization. The tissue collection took no more than 5 min, and the analysis took around 2 min from sampling.

Because there was no pretreatment during the analysis of the samples, an extremely complex metabolite profile was expected.[32] More than 1000 peaks were detected from a sample of 1−5 pL (Figure 1). All peaks were analyzed, evaluated, and exported for statistical analysis. The normalized data were classified into three sets (leaf set, stem set, and petal set) and were evaluated by *t* test and PCA (Figure 2). Statistical results are shown in Table 1. We could confirm by PCA that the collected data were classified to each site and that it is valid to study peaks which are specific to a particular site of sample.

In contrast, the *t* test was used to compare the intensities of two sets of MS data. The peaks that are found in only the leaf cell or only the stem cell correspond to *t*-values of 100% and −100%, respectively. The *t*-value is around 0% if the peak is found in both samples with similar intensity. This analysis helps to differentiate peaks and to find molecules that are specific for one site of tissue (Table 1).

■ RESULTS AND DISCUSSION

After data processing, m/z values were selected that gave a high *t*-value for the peaks. The selected m/z values, the proposed chemical formulas, and molecular identities are shown in Table 2. Molecular identification was performed by using a combination of high-resolution mass data, MS/MS data, and database results (e.g., PubChem, MassBank, and the Dictionary of Natural Products).

One example of these specific molecules is the peak m/z 169.12223 (Δppm −0.45), which had a high *t*-value (Table 1) and was identified in its protonated form $[M + H]^+$ as geranic acid (3,7-dimethyl-2,6-octadienoic acid, $C_{10}H_{16}O_2$). Another example is the peak m/z 185.15355 (Δppm −0.36), which was identified in its protonated form $[M + H]^+$ as methyl citronellate (methyl 3,7-dimethyloct-6-enoate, $C_{11}H_{21}O_2$). Following this procedure, other compounds from metabolism of geraniol, monoterpene, and terpenoid were detected (Table 1). The *t* test diagrams in Figure 3 show that geranic acid was specific to the leaf, whereas methyl citronellate was mainly found in both the leaf and stem but was scarce in the petal. This information could be valuable for comparing different cells and gives a qualitative picture of the chemical composition of each set of samples. These molecules are supposed to be barely detectable by electrospray ionization (ESI). Although proto-

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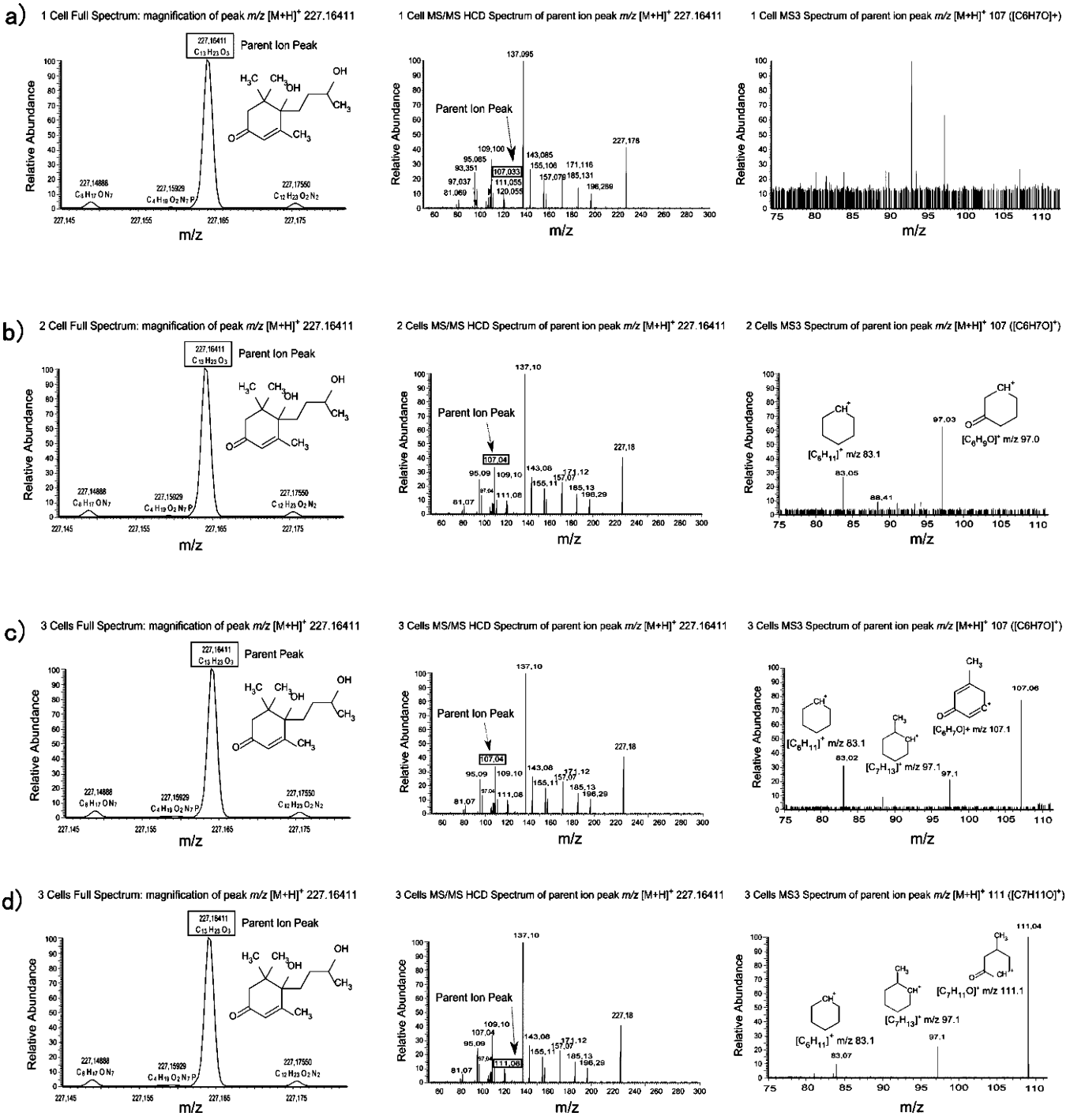

Figure 4. (a) Leaf-single-cell full spectrum magnification of peak m/z 227.1 (blumenol B), MS/MS HCD single-leaf-cell spectrum of parent ion peak m/z 227.1, and low-definition CID single-leaf-cell MS^3 spectrum of the MS/MS parent ion at m/z 107 ($[C_6H_7O]^+$). (b) Two-leaf-cell full spectrum magnification of peak m/z 227.1 (blumenol B), MS/MS HCD two-leaf-cell spectrum of parent ion peak m/z 227.1, and CID two-leaf-cell MS^3 spectrum of the MS/MS parent ion peak at m/z 107 ($[C_6H_7O]^+$), which gave a product ion at m/z 83 ($[C_6H_{11}]^+$). (c) Three-leaf-cell full spectrum magnification of peak m/z 227.1 (blumenol B), MS/MS HCD three-leaf-cell spectrum of parent ion peak m/z 227.1, CID three-leaf-cell MS^3 spectrum of the MS/MS parent ion at m/z 107 ($[C_6H_7O]^+$), which gave product ions at m/z 97 ($[C_7H_{13}]^+$) and m/z 83 ($[C_6H_{11}]^+$). (d) Three-leaf-cell full spectrum magnification of peak m/z 227.1 (blumenol B), MS/MS HCD three-leaf-cell spectrum of parent ion peak m/z 227.1, CID three-leaf-cell MS^3 spectrum of the MS/MS parent ion at m/z 111 ($[C_7H_{11}O]^+$), which gave product ions at m/z 97 ($[C_7H_{13}]^+$) and m/z 83 ($[C_6H_{11}]^+$).

nated positive monoterpene ions have been weakly detected by ESI,[33] this seems to be because of the partial corona discharge from the top of the nanoelectrospray needle to the inlet of the spectrometer, which induces atmospheric pressure chemical ionization.[34]

MS/MS analysis was performed to confirm the elemental composition of the compound peaks, and the results are shown in Table 2. Examples of the MS/MS spectra for geranic acid and methyl citronellate and their structure indicating the fragment ions are shown in Figure 3. The HCD mode with a normalized collision energy from 55% to 85% was used instead

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of CID because a higher number of product ions were visible in the HCD mode, which allows better compound identification. Authentic standards for all the compounds identified in this work (0.01 mM and 1 μM) were used to confirm the results.

Although the high resolution of the Orbitrap mass spectrometer used here was beneficial,[35] it was difficult to identify the compounds because of the high number of structural isomers with the same molecular weight and formula (Tables 1 and 2). When theoretical elemental compositions are calculated using the most common biological elements (carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus), only a limited number of combinations of atoms will accurately match a particular m/z. Molecules of the same nominal mass and different molecular formulas can be distinguished using calculated accurate masses (Da).[36,37] Usually less than Δppm ±2.5 is the criteria for exact mass molecular identification; however, we set this to less than 1 ppm. This principle was used to identify our compounds. However, when the molecular weights and formulas are exactly the same, as in structural isomers, and many of them belong to known metabolic pathways, additional information is required to assign possible structures. MS/MS and MS^3 are useful techniques for obtaining structural information from fragmentation pathways that result in different product ions with different intensities that can be used to identify products using MS databases. Techniques such as chromatography are also helpful for identifying isomers. Although single-cell MS/MS analysis was successful, single-cell MS^3 analysis was not initially successful because of the low amount of sample. In order to solve this problem, MS^3 analysis was carried out on samples of two and three cells from the same tissue with similar morphology. The signals of the MS/MS and MS^3 spectra became higher by taking advantage of ion trap capacity of the spectrometer at selected ion monitoring (SIM), and the three cell MS^3 analyses gave a strong enough signal to generate spectra (Figure 4c,d).

MS^3 experiments were performed with mixtures of the standards at different relative concentrations but at total concentrations which were similar to those used for MS/MS analysis, however, the MS^3 spectra could not distinguish structural isomers. Nevertheless, the three-cell MS^3 experiments were useful for identifying some molecules. Among the peaks that were found specifically in petal cells was the m/z 227.164 11 (Δppm −0.27, $C_{13}H_{23}O_3$) peak (Table 1). Further MS/MS analysis (Table 2) limited the search of the compounds, which fitted the molecular formula and MS/MS spectra pattern to two possibilities: methyl dihydrojasmonate (methyl(3-oxo-2-pentyl-cyclopentyl)-acetate) and blumenol B ((6S,9R)-6,9-dihydroxymegastigman-4-en-3-one) in their protonated form $[M + H]^+$. A methyl dihydrojasmonate standard was compared with the unknown compound, and the two spectra did not match. Thus, the metabolite was identified as blumenol B in its protonated form (Figure 4a).

The norisoprenoids blumenol A and blumenol B are important components in the flavor of tobacco,[38] tea, and some fruits. They have been previously identified in many other plants,[39] although their biogenetic origin is as yet unknown.[40] To our knowledge, blumenol B has never been reported in *Pelargonium zonale* (*Pelargonium x hortorum*).

The stereochemistry of blumenol B has previously been identified by techniques such as NMR, by chemical conversion from related compounds, and application of the modified Mosher's method[41] or by synthesizing the compound for use as a standard. Single-cell MS^3 analysis was not successful because of the low signal intensity of the compound peaks in the sample (Figure 4a). Figure 4 shows that the definition of MS/MS and MS^3 spectra increased as the number of cells extracted increased.

The CID three-leaf-cell MS^3 spectrum of the parent ion at m/z 107 ($[C_6H_7O]^+$) (Figure 4c) and the spectrum of the parent ion m/z 111 ($[C_7H_{11}O]^+$) (Figure 4d) found in the MS/MS spectra results show product ions at m/z 97 ($[C_7H_{13}]^+$) and m/z 83 ($[C_6H_{11}]^+$), indicating the loss of both a carbonyl group and a methyl group. These spectra provide useful information for structural identification because of the presence of characteristic product ions and support the assignment of this compound as blumenol B.

CONCLUSIONS

The method we presented in this paper is a tool for identifying specific molecules in single plant cells and for locating them in different tissues. It will allow the comparison of different plant cell types in undamaged tissues and could be used as a complementary tool for plant metabolomics and molecular identification. However, further development is needed in order to enable differentiation of structural isomers and accurate quantification of compounds in the samples. We expect that this analytical method will be useful in a wide variety of practical applications, such as rapid quality control of crops, analysis, treatment and control of plant diseases, food analysis, quality control of medicinal plants, and metabolism studies of bioactive compounds in medicinal plants.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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