Spatially Resolved Plant Metabolomics: Some Potentials and Limitations of Laser-Ablation Electrospray Ionization Mass Spectrometry Metabolite Imaging

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Laser-ablation electrospray ionization (LAESI)-mass spectrometry imaging has been applied to contrasting plant organs to assess its potential as a procedure for performing in vivo metabolomics in plants. In a proof-of-concept experiment, purple/white segmented Petunia spp. petals were first analyzed using standard liquid chromatography-mass spectrometry analyses of separate extracts made specifically from the purple and white regions. Discriminatory compounds were defined and putatively annotated. LAESI analyses were then performed on living tissues, and these metabolites were then relocalized within the LAESI-generated data sets of similar tissues. Maps were made to illustrate their locations across the petals. Results revealed that, as expected, anthocyanins always mapped to the purple regions. Certain other (nonvisible) polyphenols were observed to colocalize with the anthocyanins, whereas others were found specifically within the white tissues. In a contrasting example, control and Cladosporium fulvum-infected tomato (Solanum lycopersicum) leaves were subjected to the same procedures, and it could be observed that the alkaloid tomatine has clear heterogeneous distribution across the tomato leaf lamina. Furthermore, LAESI analyses revealed perturbations in alkaloid content following pathogen infection. These results show the clear potential of LAESI-based imaging approaches as a convenient and rapid way to perform metabolomics analyses on living tissues. However, a range of limitations and factors also have already been identified that must be taken into consideration when interpreting LAESI-derived data. Such aspects deserve further evaluation before this approach can be applied in a routine manner.

Plants are a tremendously rich source of a myriad of structurally and chemically diverse metabolites (Rao and Ravishankar, 2002; D’Auria and Gershenson, 2005). Many of these metabolites have a (partly) known function in the plant, although our knowledge of the vast majority of plant secondary metabolites is still sparse, or even nonexistent (Rao and Ravishankar, 2002; D’Auria and Gershenson, 2005; Fernie, 2007). Plant metabolites are also of considerable importance in a crop context. Indeed, most plant species that have undergone domestication have become crops specifically because they provide us with a source of chemicals. This is not only true for all of our food crops, but also for many other species of genera such as Pyrethrum (insecticides), Jasminium and Santalum (perfumes), Hevea (rubber), Nicotiana and Cannabis (drugs), Linum (oils), Artemisia and Taxus (pharmaceuticals), Cinnamomum (flavors), etc. However, despite the importance of plants as a source of exploitable and essential biochemicals, we often still have remarkably limited knowledge of the relevant biosynthetic pathways, the genetics behind the key enzymes, and indeed when, why, and where these metabolites are produced and stored within the plant in question (Fernie, 2007; Sumner et al., 2011; Kueger et al., 2012).

The field of plant metabolomics has grown tremendously since its recent inception earlier this century (Fiehn et al., 2000; Fiehn, 2002). As an untargeted approach to gain a broad overview of the complexity of plant metabolic composition, the technology has, in a short time, made significant inroads into helping expand our knowledge of plant biochemistry (Kueger et al., 2012; Etalo et al., 2013; Hunerdosse and Nomura, 2014; Meret et al., 2014). Typically, rich metabolomics data sets already provide us with a valuable means to generate hypotheses relating to plant metabolism, which then become the focus of further, more direct investigation (Quanbeck et al., 2012). New technologies are being developed, and especially, new data-mining strategies are being designed to allow us to look deep into plant metabolism without having first to rely on preconceptions. However, there are significant limitations to the application of the technology, which still remain the topic of much research effort.

Robust sampling approaches for plant biochemical analysis generally entail taking reliably measurable findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Robert D. Hall (robert.hall@wur.nl).

R.D.H. conceived the project; D.W.E. executed the experiments, analyzed the data, and produced the figures; R.C.H.D.V. was involved in metabolite identification and data discussions; M.H.A.J.J. was involved in the tomato–Cladosporium experiment; all authors were involved in designing the experiments and writing the article.

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Arms and Gershenson, 2005).

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amounts of plant material that will yield detectable levels of the chemical components. Although for metabolomics analyses, samples of just 50 mg can often suffice, obtaining a reliable sample with minimum biological variation generally requires an initial pooling of materials from which a representative sample is then taken. We therefore treat plant tissue as being homogeneous, but this is clearly a gross oversimplification (Fernie, 2007). Plants have been considered to be composed of roughly 40 different cell types, and a plant organ such as a leaf will generally contain up to 15 different cell types (Martin et al., 2001). Different morphologies also parallel different biochemical composition. Even directly neighboring cells within an organ, for example, a leaf epidermis that often comprises pavement, guard, trichome, and glandular hair cells, are formed from cells already known to have distinctly different biochemistries. Making an extract, for any kind of metabolomics or standard biochemical analysis, therefore entails that we immediately lose most intercellular and intertissue resolution. However, our knowledge is growing in that, in addition to known or expected biochemical differences between cell types, metabolite accumulation across organs can be far from uniform; indeed, islands of higher and lower concentrations of particular metabolites have been observed. This is of course immediately visible when the metabolites concerned can be seen by the naked eye; anthocyanins, for example, are often found to be heterogeneously distributed across leaves, fruits, and flower petals, creating clear phenotypic patterns. The same may also be true of other compounds that are invisible to the human eye but that, in contrast, may still be detectable by insects (e.g. through their fluorescence capacity; see http://www.naturfotograf.com/UV_flowers_list.html; Gronquist et al., 2001).

In an ideal situation, we would like to be able to look directly into a plant tissue and be able to analyze the biochemical composition at the single cell level. Some so-called metabolite imaging technologies, usually based on mass spectrometric detection (mass spectrometry imaging [MSI]), have recently been introduced as a step toward this optimistic goal. Included here are matrix-assisted laser desorption/ionization (MALDI)-MSI, direct analysis in real time, and desorption electrospray ionization approaches (Cody et al., 2005; Cornett et al., 2007; Ifa et al., 2010). Early examples of MALDI-MSI have shown not only how primary metabolites such as sugars can be strongly localized within plant organs (Rolletschek et al., 2011), but also how the heterogeneous distribution of glucosinolates in Arabidopsis (Arabidopsis thaliana) can potentially determine grazing behavior of caterpillars (Shroff et al., 2008). This technology continues to improve, and recent exciting developments have resulted in cellular and subcellular imaging of metabolites at a resolution of 5 to 9 μm using MALDI (Korte et al., 2015). However, some key practical limitations of MALDI-based approaches are centered around the need to initially have to pretreat/dehydrate the tissue prior to applying the required matrix solution and the requirement of applying a vacuum during the biochemical analysis. Recently, a new technology has been introduced, laser ablation electrospray ionization (LAESI), which can potentially overcome some of these limitations, given that measurements can be made on fresh, living tissue without the need for a vacuum, thus creating the potential for high-resolution in vivo metabolomics.

Here, we report on a set of experiments performed to assess both the potential and limitations of using LAESI-based MSI approaches to perform metabolic mapping on living plant tissues. While identifying a number of technological challenges that still need to be tackled, we were able to show that it is possible to use LAESI-MSI to map metabolites directly onto their known location (in this case, by exploiting the visibility of anthocyanins) as well as localize invisible metabolites in the same tissue. Results have revealed that in plants, for both petal and leaf tissue, the distribution of metabolites can be highly heterogeneous, and that this heterogeneity is of potential relevance to our gaining a broader, more detailed understanding of the overall molecular organization and phenotypic features of plant tissues. Furthermore, knowledge of the nature and extent of this heterogeneity has particular relevance and importance when trying to understand how a plant functions as a system, interacting with its environment. We predict that a higher resolution understanding of plant biochemistry will lead to an increasingly discriminatory capacity in our ability to define more accurately the spatial complexity of plant molecular organization.

**RESULTS**

In preparation for the LAESI-mass spectrometry (MS) analysis, standard comparative metabolomics analyses were initially performed on the two contrasting tissue sources chosen for use in this study, in relation to their associated biological and technological challenges. These analyses involved liquid chromatography (LC) coupled to an Orbitrap Thermo Fourier Transform Mass Spectrometry (FTMS; LC-MS) to record accurate masses, combined with photodiode array (PDA) detection to determine the UV/Visible (Vis) spectra of the eluting compounds. First, we used Phal- eanopsis spp. flowers to detect and identify semipolar secondary metabolites associated with the variegation in petal color. Both purple and white regions from these orchid petals were dissected using a cork borer (macrodisssection), and their metabolites were extracted and compared in an untargeted manner using full-scan LC-MS profiling. Following this global LC-MS-based comparative metabolomics, the most clearly differentially accumulating metabolites were subsequently identified using tandem mass spectrometry (MS/MS) in combination with UV/Vis spectral analysis. Spatial mapping of these differential metabolites in intact petals was then performed using LAESI-MS.

For tomato (Solanum lycopersicum), again, we first monitored the changes in secondary metabolites, and
specifically the glycoalkaloids, in crude extracts of control and *Cladosporium fulvum*-infected leaves of susceptible cv Moneymaker-Cf-0 lines using untargeted LC-MS. This compatible plant-pathogen interaction was selected based on our previous work where it was shown that glycoalkaloids, which are toxic to *Cladosporium* spp., are degraded by the fungus upon inoculation of susceptible tomato plants (Ökmen et al., 2013). To perform spatial mapping of these biotic changes using LAESI-MS, we monitored the local, late-infection-associated (16 days post inoculation) change in the fungitoxic glycoalkaloid α-tomatine after performing point inoculations using fungal spore suspensions.

To perform LAESI analyses on the orchid petals and on tomato leaves, these organs were directly mounted onto the x-y-z sample stage, and the tissues were then subjected to intense infrared laser pulses, ablating metabolites from the entire tissue thickness, followed by ionization of the ablated compounds by an electrospray plume and subsequent detection of positively charged ions by an accurate mass Quadrupole Time of Flight MS (Fig. 1). All analyses were thus performed on living tissues that had not undergone any pretreatment and under ambient conditions. This system enabled us to detect hundreds of metabolite ions and determine their distribution and relative abundance at a spatial resolution of 200 μm.

**Purple Regions of *Phalaenopsis* spp. Petals Are Enriched with Cyanidin-Type Anthocyanins**

Global LC-MS analysis of extracts from the macro-dissected orchid petals indicated differential abundance of a series of semipolar secondary metabolites in both the pigmented and nonpigmented regions (Supplemental Fig. S1). Most of the metabolites that were readily detected within the purple patches were below detection limits in the white areas of these petals (Supplemental Fig. S1). In contrast, most of the metabolites that are abundant in the white patches were also detected in the purple part, but at a lower abundance. Based on this prior information, we performed LAESI-MS analysis on the orchid flowers to spatially map a number of selected metabolites associated with

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**Figure 1.** A schematic representation of LAESI-MS. Under ambient atmospheric conditions, water-containing tissues such as plant leaves are subjected to mid-IR (2,940 nm) laser pulses. The absorption of such an intense laser pulse by the sample produces small bubbles within the sample without ion formation. When the bubble explodes, a jet of vaporized material (ablated sample) is ejected above the sample surface without ionization. The ablated sample then intersects a stream of ions from an electrospray source (electrospray plume), which ionizes the molecules of the jet (ionized sample). The ionized molecules subsequently are directly drawn into the mass spectrometer for analysis. Using Protea Plot software for data analysis, the detected mass features can subsequently be converted into two-dimensional or three-dimensional ion maps and can be overlaid on the original sample image.
(non)pigmentation in situ. In line with the LC-MS analysis, the LAESI-MS analysis clearly indicated the localization of particular mass features in the purple regions of orchid petals (Supplemental Fig. S1).

For identification of those particular compounds that were specific to the purple or white patches of the petal, priority was given to mass features that could be commonly detected by both platforms (LC-MS and LAESI-MS; Supplemental Fig. S1). In these analyses, a range of flavonoids (i.e. anthocyanins and flavones) were the dominant metabolites that showed specific localization to either the purple or the white areas of the tissue (Supplemental Fig. S1). Almost all detected anthocyanins that were common to both platforms (molecule proton adduct [M+H]⁺; mass-to-charge ratio (m/z) 817.2183 [C₃₈H₄₁O₂₀], m/z 1185.3291 [C₅₅H₆₁O₂₉], m/z 1271.3303 [C₅₉H₆₃O₃₂], and m/z 1287.3615 [C₅₉H₆₇O₃₂]) could be identified as cyanidin conjugates, based on the fact that they show both a PDA absorbance maximum at approximately 520 nm and the specific MS/MS fragment of m/z 287.0550 corresponding to C₁₅H₁₁O₆ within 3-ppm accuracy (Fig. 2; Supplemental Figs. S1 and S2).

Hierarchical cluster analysis based on the LC-MS data suggested that these anthocyanins are biochemically closely related (Supplemental Fig. S1). MS/MS-based structural analysis further indicated that the anthocyanins are decorated to various degrees with glycosides as well as malonyl and coumaroyl groups (Fig. 2; Supplemental Fig. S2). In both LC-MS and LAESI-MS analyses, flavones were the major semipolar metabolites that colocalized with these anthocyanins; in particular, luteolin-type flavonoids were the dominant form (Supplemental Fig. S1). Although the purple pigmentation appears to be uniform, as observed with the naked eye, the abundance of the cyanidins within the purple-pigmented patches was characterized by a gradient feature, indicating a nonuniform distribution of anthocyanins within the purple spots (Fig. 2).

**Luteolin-Type Flavonoids Show Colocalization with Anthocyanins**

Two luteolin-derived flavonoids ([M+H]⁺; m/z 449.1077 [C₂₁H₂₁O₇] and 611.1603 [C₂₇H₃₁O₁₆]) appeared, like the cyanidins, to be significantly different between the LC-MS profiles of dissected purple and white petal areas (Supplemental Fig. S1). Their structures were elucidated based on both their MS/MS fragmentation patterns (Supplemental Fig. S2) and their UV/Vis spectrum showing an absorbance maximum at 350 nm and with a second peak at 255 nm, corresponding to luteolin-based structures (Plazonič et al., 2009). The primary difference between these two flavone species was their number of glycosidic moieties (Fig. 3). The C-glycoside type of conjugation in both molecules was evident from the typical fragmentation pattern of the parent ions, which showed i,jX fragments accompanied by successive losses of water molecules due to intraglycosidic cleavages (Supplemental Fig. S2). Although the type of sugar is not directly distinguishable from the mass spectra, the predominant sugar form was a hexose, corresponding to losses of m/z 162 D (Supplemental Fig. S2). The LAESI-MSI analysis clearly

![Image](https://example.com/figure2.png)

**Figure 2.** LAESI-MS imaging showing the specific accumulation of cyanidin-type anthocyanins in purple patches of a *Phalaenopsis* spp. petal. The molecular structures associated with the ion maps were predicted from LC-MS/MS fragmentation analysis of samples obtained from the purple patches of the petals. The false color associated with the ion map represents the base peak intensity (BPI) of [M+H]⁺ masses at a 10-ppm window. The first image on the left depicts a laser-ablated orchid petal in ambient light. Each small black dot marks the position of an ablation point, with a diameter of 200 µm and a separation distance of 500 µm. The remaining four images depict the mapped locations of four contrasting m/z ions as detected using LAESI analyses. High-resolution LC-MS/MS spectra and UV absorption maxima were used to make predictions of the chemical group and the molecular structures in each case.
indicated that the luteolin derivatives colocalize with the cyanidins within the purple patches of these Phalaenopsis spp. petals (Fig. 3) and are undetectable in the white petal tissues. As was the case with the anthocyanins, the distribution of these flavonoids within the purple patches was characterized by clear concentration gradients, indicating a nonuniform distribution (Fig. 3).

**Apigenin-Based Flavonoids Show an Inverse Spatial Distribution**

In contrast to the anthocyanins and luteolin-derived flavonoids in the purple patches, apigenin-containing flavonoids were specifically abundant in the white areas of the orchid petals (Fig. 4). The two flavones identified ([M+H]^+ m/z 565.1548 [C_{26}H_{29}O_{14}] and 741.2027 [C_{36}H_{37}O_{17}]) both harbor a C-glycosylated pentose and an O-glycosylated hexose, with an m/z of 741.2027 harboring an additional feruloyl group (Fig. 4; Supplemental Figure S2). These metabolites showed an absorption maximum at 332 nm with a second peak at 260 nm. This, together with the C_{15}H_{11}O_{5} MS/MS fragment, corresponds to apigenin-derived flavonoid structures (Amat et al., 2009).

**LAESI-MSI Reveals Dramatic Depletion of α-Tomatine in Tomato Leaves at the site of C. fulvum Inoculation**

Unlike the visible heterogeneous distribution of anthocyanins in Phalaenopsis spp. petals, the major biochemical changes associated with plant biotic stress, such as pathogen infection, are generally not visible to the naked eye. However, several studies have indicated that substantial metabolic reprogramming does take place. The LC-MS analysis of extracts prepared from homogenized leaves of susceptible tomato plants infected with C. fulvum revealed clear alterations in the global metabolic profiles (Fig. 5). This was particularly evident from fungus-mediated conversion of the major antifungal glycoalkaloid α-tomatine into its nontoxic aglycone tomatidine, confirming our earlier work (Ökmen et al., 2013; Etalo et al., 2013). Using LAESI-MS of intact leaves of the same susceptible tomato plants 16 d after point inoculation with C. fulvum spores, we were able to show a very localized and strong reduction in the level of α-tomatine toward undetectable levels at the site of C. fulvum inoculation (Fig. 5). Interestingly, LAESI-MS also revealed that the distribution of α-tomatine in the unchallenged control leaf of tomato is not evenly distributed, but rather it is, once again,
characterized by a patchy distribution pattern. The same is true for other metabolites that were less affected by the presence of the fungus (Fig. 5).

**Some Limitations Associated with LAESI-MS for Biological Sample Analysis**

In the course of our experiments, a number of technological challenges and limitations relating to the use of the LAESI-MS approach have become apparent and need to be taken into consideration regarding the experimental design, data analysis, and interpretation of the results. These aspects are currently under investigation.

**Tissue Water Content**

LAESI-MS has been designed for and is particularly well suited to the investigation of water-containing specimens (Nemes and Vertes, 2010). The water content of the tissue is one of the major factors affecting the ablation characteristics and the success of vaporization/ionization of the material. Hence, care must be taken when interpreting results from tissues where the water content might differ significantly, either naturally or as a result of treatment. This is important, for example, when comparing materials under abiotic stress conditions, genotypes with significantly different anatomies/morphologies, or different tissue types. To demonstrate this, water was vacuum infiltrated into tomato leaves, whereas control leaves were subjected to the same degree of vacuum, but in the absence of water. When analyzed using LAESI-MS, tomato leaves infiltrated with water showed a significant overall increase in the intensity of the detected mass features (Fig. 6). Furthermore, the comparative localized biochemical changes between *C. fulvum*-inoculated and control tomato leaves are substantially clearer when the leaves have been infiltrated with water prior to LAESI-MS (Fig. 6).

**Inability to Separate Isomeric and Isobaric Compounds**

Unlike in LC-MS analyses, when using LAESI-MS, isomers and isobars are not easily distinguishable. As an example, it is worth examining data for a few of the metabolites, such as glycoalkaloids and flavonoids that were detected in both plant species used here. In positive ionization mode, the tomato glycoalkaloid α-tomatine is
characterized by partial in-source fragmentation in the FTMS, resulting in the generation of fragment ion masses corresponding to both the aglycon tomatidine and some of its glyco conjugates. During LC-MS analysis of tomato leaf extracts, the exact mass of tomatidine derived from in-source fragmentation of \( \alpha \)-tomatine can be easily discriminated from that derived from the molecular ion tomatidine, as they can be chromatographically separated (Fig. 7). However, in full-scan LAESI-MS analysis, which essentially resembles a direct injection of a crude extract, distinguishing similar accurate masses derived from contrasting sources (in this example, \( m/z \) 416.3524 from the ionization of the endogenously present aglycon molecule and the in-source fragmentation of its glycoside) is impossible due to the lack of separation potential before entering the MS. For the same reason, masses from different isomers or isobars that can frequently be separated by LC-MS are indistinguishable when using the LAESI-MS approach. In this analysis, the apigenin-related flavonoids are good examples. In our LC-MS analysis, we detected four different molecules with \( m/z \) 565.1548 (Supplemental Fig. S3). However, in the LAESI-MS analysis, only one generic form of this mass was detectable.

**Ion Suppression**

Another potential limitation for all analytical MS systems without a hyphenated extensive separation
methodology is ion suppression. The lack of any chromatographic separation in a LAESI-MS approach, therefore, also renders this technique liable for ion suppression effects. To demonstrate this, the synthetic peptide Leu enkephalin (LE; [M+H]+ m/z 566.2771) was mixed in the electrospray solution that was used to form the plume during the LAESI operation (Fig. 1). We used the analysis time pattern of the base peak intensity of the alkaloid phalaenopsine, the most detectable metabolite in the orchid petals, to demonstrate the influence of the sample matrix on the detector response for LE while it is continuously introduced into the system. This analysis showed that the signal level of LE added to the spray solution inversely corresponded to the signal intensity of phalaenopsine coming from the tissue (Fig. 8), thus indicating a direct ion suppression effect of the plant matrix on LE detection. This is of particular importance when quantification is an issue.

DISCUSSION

In this work, we have shown the potential and some of the limitations of the LAESI-MS technique when used for in situ metabolite mapping specifically in plant research. With the advancement in molecular biology techniques and the utilization of, for example, reporter genes, we are now able to monitor with precision the localization and expression of gene(s), even in real time in a range of plant species. This often highly localized expression, which can be related to cell, tissue, or organ specificity as well as being a potential result of external signals, can now be monitored from specific tissues down to even individual subcellular compartments (Wiśniewska et al., 2006; Soriano et al., 2014; Zhang et al., 2014). However, with the exception of the small number of visible metabolites (pigments), an equivalent broad ability to perform similar in situ analyses at the metabolite level has lagged far behind. Although we have been able to localize a few metabolites using various histochemical or fluorescent probes, the application of these techniques to a wide range of metabolites, simultaneously, in living tissues was inconceivable up until now.

Through LAESI-MS analyses, hundreds of metabolites can be detected at a spatial resolution of 200 μm, which is comparable with a spatial resolution of approximately 4 plant epidermal cells. Using orchid petals for a proof of principle, and by exploiting the visibility of anthocyanins, we have been able to confirm the resolution capacity of this approach for metabolite mapping. We have shown that the spatial localization/colocalization of anthocyanins to specific tissue regions of the petals (pigmented...
versus nonpigmented) matches the expected, visible pattern. Furthermore, it has also been possible to demonstrate colocalization of molecules in the pigmented parts of orchid petals, not only involving different cyanidin anthocyanin derivatives, but also, and most importantly, colocalization of molecules that are not visible to the naked eye. Our work has also allowed us to find metabolites specific for the nonpigmented flower petal regions, even involving metabolites chemically related to those in the pigmented regions, thereby strongly demonstrating the broadness and complexity of the localization of metabolites within plant tissues and organs.

This phenomenon of biochemical colocalization is in line with the proposed π-stacking interactions of anthocyanins with themselves (self association), for which the function is still currently unknown (Di Meo et al., 2012; Fernandes et al., 2015). Furthermore, π-stacking interactions can also occur within the pool of colorless polyphenols (flavones, flavonols, and hydroxycinnamic acids) that can coexist with anthocyanins in the vacuole, a phenomenon referred to as copigmentation (Di Meo et al., 2012). Interestingly, in our spatial imaging analysis of Phalaenopsis spp. petals, luteolin-related flavones are the most prominent metabolites colocalizing with the anthocyanins. LAESI analysis has, therefore, enabled the visualization of the spatial distribution of multiple secondary metabolites, and not just the anthocyanins that are associated with a specific pattern of pigmentation in the orchid petal. This technique has clearly demonstrated the strong spatial heterogeneity of metabolites in plant tissues and can help us to visualize metabolites that were invisible until now.

In the specific case of Phalaenopsis spp. flowers, the inverse spatial distribution of luteolin- and apigenin-related flavonoids in the purple and white parts of the petal, respectively, is potentially an indication of a strong localized activity of flavonoid 3′-hydroxylase, an enzyme involved in hydroxylation of the flavone substrates for flavone synthase, thus determining the accumulation of apigenin versus luteolin type of flavones. This signifies the potential power of spatial imaging MS not only in the identification and spatial mapping of metabolites, but also in allowing the identification of important biochemical and genetic components that are strongly correlated with the observed chemical phenotype under investigation. Further research, involving, for example, tissue- or cell level-based gene expression analysis (Brandt et al., 2002), would be a logical next step to decipher important genetic components associated with the observed chemotype and help us home in on and find the specific genes and promoters responsible for the phenotypic heterogeneity.

Beyond the proof of concept, involving naturally occurring differences in spatial distribution of visible plant metabolites such as anthocyanins, our work on tomato leaves demonstrated that LAESI-MS also allows us to monitor other biological and biochemical phenomena, such as a plant’s response to biotic stress. In this example, we were able to observe metabolic perturbations through the visualization of pathogen-mediated changes in glycoalkaloids in C. fulvum-inoculated tomato. Such perturbations in the abundance of metabolites lacking chromophoric groups that make them visible would be otherwise almost impossible to observe in situ at this degree of resolution. Interestingly, the control analyses of uninfected material immediately revealed that even under normal, healthy growth conditions, the distribution of the alkaloid α-tomatine and other (unrelated) metabolites is highly heterogeneous.
showing distinct patching (islands) across the leaf. Similar metabolic heterogeneity has been observed previously when using lower resolution imaging systems such as MALDI and desorption electrospray ionization (Shroff et al., 2008; Li et al., 2013). Together, these observations indicate how the distribution and abundance of plant metabolites in a tissue are often nonuniform. However, conventional analytical techniques required for robust sampling must treat plant tissues as being homogeneous, which is clearly incorrect. This implies that, by using such conventional methods, we lose a significant degree of resolution in our understanding of the spatial-temporal heterogeneity of in vivo plant metabolite biosynthesis and accumulation.

Clearly, the potential of LAESI-based metabolite imaging approaches to provide a window into the distribution of plant metabolites, as demonstrated here, is considerable and hugely exciting. However, there are still clear challenges and limitations that warrant further study and assessment, some of which, such as multiple adduct formation (K, Na), of course are not just specific to LAESI approaches. Sample architecture (flatness, presence of protruding structures such as veins, etc.) and strong differences in cellular structure need to be taken into consideration when comparing samples. This is of particular relevance when comparing treatments that have a significant structural effect on plant phenotype. However, the variation in water content, the tensile strength of the tissue, and the lack of chromatographic separation prior to the MS are some of the main limitations we have encountered. Specifically, this could be visible while comparing the metabolic contents of strongly contrasting tissues or equivalent tissues that have undergone treatments with significant physical or physiological effects. Hence, both experimental design and any interpretation of LAESI-MS results should be performed with caution. In general, further verification of the results with conventional LC-MS or other analytical techniques is required, particularly in relation to metabolite identification/tracking. However, LAESI approaches complemented with LC-MS analysis of metabolite extracts of macrodissected or laser-microdissected tissue offer a wonderful opportunity and can, in combination, be very powerful. Using conventional analytical techniques to help locate and identify discriminatory compounds potentially linked to phenotypic and behavioral differences, and then applying LAESI-MS to determine the exact spatial distribution and the degree of potential physical/temporal heterogeneity of these discriminatory compounds (as it was performed in this study) is a robust and reliable way to proceed.

Monitoring of the detector response for the nonplant marker compound LE present in the spray solution pointed at another drawback of LAESI: the occurrence of ion suppression by the sample matrix. What this implies is that the abundance of individual metabolites may be underestimated; significant alterations to the composition of different areas of the plant matrix could potentially result in misleading conclusions. Highly abundant and well-ionizable molecules have the power to suppress less-ionizable and less-abundant molecules. This is of course nothing new regarding approaches using direct injection methodologies (Müller et al., 2002), and therefore, the same caution and conciliatory measures should be taken regarding LAESI-MS data interpretation. Although ion mobility MS can provide a potential solution to resolving isomers in direct injection analyses, the resolution of similar ions that are produced through in-source fragmentation will remain as yet an insurmountable challenge. The simultaneous occurrence of the same ions from different origins in the ionization chamber prevents any level of resolution. Once again, a valid solution to this problem is the use of combined LAESI with LC-MS. An LC-MS analysis can provide both qualitative and (semi)quantitative information about the metabolites in our sample; subsequently, LAESI-MS analysis can be applied effectively to generate semiquantitative information on the spatial distribution of metabolites throughout the tissue under examination.

In conclusion, these results have revealed that in plants, for both petal and leaf tissue, the distribution of metabolites can be highly heterogeneous, and that this heterogeneity is of potential relevance to gaining a broader, more detailed understanding of the overall molecular organization and phenotypic features of plant tissues. Furthermore, knowledge of the nature and extent of this heterogeneity has particular relevance and importance when trying to understand how plants function as a system interacting with their environment. We predict that a higher-resolution understanding of plant biochemistry will lead to an increasingly discriminatory capacity in our ability to better define the spatial complexity of the plant molecular organization.

MATERIALS AND METHODS

Reagents and Sample Materials

Ultra LC-MS-grade methanol and water were obtained from Acto-All Chemicals. Formic acid was from Biosolve Chemicals. Fresh (nonfrozen) sample materials were used for the analysis. Flowering orchid (Phalaenopsis spp., hybrids) plants were purchased from a local garden center. Tomato (Solanum lycopersicum; ‘Moneymaker’ CF5) plants were germinated and grown from seed under standard greenhouse conditions, and 3-week-old plants were used for analysis. For inoculation with fungal spores, essentially a 2-μL aliquot of spore (2 × 10⁷) suspension of Cladosporium fulvum was placed onto the abaxial surface of the leaf, after which the plants were placed in a high-humidity chamber in the greenhouse for 3 d.

LAESI-MS Analysis

A Protea Biosciences model DP-1000 LAESI system was coupled to a Waters model Synapt G2S mass spectrometer. The LAESI system (Fig. 1) was equipped with a 2,940-nm mid-infrared laser yielding a spot size of 200 μm; the laser was set to fire 10 times per x-y location (spot) at a frequency of 10 Hz and 100% output energy with a laser dwell time of 2 s. The system was also equipped with a syringe pump delivering a mixture of methanol-water-formic acid (50:30:0.1) at 2 μL min⁻¹, a New Objective model PicoTip 5-cm × 100-μm internal diameter stainless steel nanospray emitter operating in positive-ion mode at 3,800 V, and a Peltier-cooled motorized x-y-z stage (at 25°C) scanned in serpentine mode. The sampling location x-y center-to-center distance was adjusted depending on the sample type used for measurement. The focusing lens
and the sample stage Z value were tuned for each sample with the help of an in-line camera and were typically in the order of 4.3 and 20.8 mm, respectively. The LAESI was operated using LAESI Desktop Software v.2.0.1.3 (Protea Biosciences). The Time of Flight mass analyzer of the Synapt G2S was operated in the V-reflectron mode at a mass resolution of 18,000 to 20,000 (full width at one-half maximum). The source temperature was 150°C, and the sampling cone voltage was 30 V. The positive ion masses acquired were in the range of m/z 100 to 2,000. Time of Flight-MS data were lock mass corrected post data acquisition using LE (m/z 556.2771), which was added to the spray solvent.

Lons of potential interest for the generation of accurate mass ion maps were identified via background subtraction of adjacent BPI chronogram regions from those BPI regions that coincided with the analog signal from the mid-infrared laser pulses, using Mass lynx software (Waters). Ion maps (10-pm window) were created in Protea Plot v.2.0.1.3 (Protea Biosciences) after importing the Mass lynx raw data files. Prior to analysis, the plant materials were firmly fixed to an appropriate carrier using sticky tape with, for Phalaenopsis spp. petals, the abaxial side exposed; and for tomato leaves, the adaxial side.

**Metabolite Extraction and Analysis by LC-PDA-LTQ-Orbitrap-FTMS (LC-MS)**

**Sample Preparation**

*Phalaenopsis* spp. Petals. Purple and white areas of orchid petals were dissected using different-sized cork borers and immediately snap frozen in liquid nitrogen. Six random petals that were obtained from different flowers were taken as separate experimental units, and for the purple and white patches, three independent replicates were prepared. Tomato leaves from the susceptible line ‘Moneymaker’-CF0 that were either inoculated with *C. fulvum* or mock (water) treated were harvested 16 d after inoculation and frozen in liquid nitrogen.

**Metabolite Extraction.** Samples were ground into a fine powder in liquid nitrogen, and 100 mg fresh weight of frozen leaf or petal powder was weighed and extracted with 300 mL of MeOH containing 0.1% (v/v) formic acid. Samples were briefly vortexed, sonicated for 30 min, and centrifuged at 21,000g for 10 min. Supernatants were filtered through a 0.2-μm inorganic membrane filter and transferred to HPLC vials for analysis.

**Metabolite Analysis Using LC-MS.** LC-MS profiling of crude plant extracts was performed using an LC-PDA-LTQ-Orbitrap-FTMS instrument consisting of an Acquity HPLC with FIA detection (Waters) interfaced to an LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source (van der Hooff et al., 2011). The sample injection volume was 5 μL. A Luna RP-C18 (2) analytical column (Phenomenex) was used for chromatographic separation. The mobile phase consisted of a binary eluent solvent system of degassed ultra-pure water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) formic acid, while keeping the flow rate of the eluents at 0.19 mL min⁻¹ and maintaining the column temperature at 40°C. The HPLC gradient started at 5% B and linearly increased to 65% B across a period of 45 min. The column was reequilibrated for 15 min following the separation of each sample (van der Hooff et al., 2011). The mass chromatograms generated by the Orbitrap-FTMS were processed (peak picking and baseline correction) in an unbiased manner using the MetAlign software package (Lommen, 2009), whereas metabolite reconstruction was performed using MScrust (Tikunov et al., 2012). From the processed data, information on the relative peak height of the representative masses [M+H]⁺ for α-tomatine and the aglycone tomatidine was extracted at their recorded retention time for tomato fruit according to the MOTO database (Moco et al., 2006) and the Komics database (http://web2.kazusa.or.jp/komics/; Ijima et al., 2008).

**Statistical and Multivariate Analyses**

For the *Phalaenopsis* spp. metabolome studies, three independent biological replicates of purple and white petals were collected from tissue regions from six petals. Similarly, four biological replicates of leaf samples from susceptible tomato plants that were either inoculated with *C. fulvum* or mock treated were used. Differentially accumulating metabolites (P < 0.05 based on two-tailed Student’s t-tests) were considered for further analyses.

GeneMaths XT (Applied Math) was used for hierarchical clustering analysis, and Pearson’s correlation coefficients were used to calculate the distance or similarity between two entries. The resulting clusters were summarized using a complete linkage algorithm. To compare metabolite levels, their relative intensity values obtained from the LC-MS data processing were log₂ transformed and scaled using the average as an offset and the SD for scaling ([x-average (offset])/SD [scale]).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Mass features and UV/Vis absorption spectrum peculiar to the purple patches of *Phalaenopsis* spp. petals as detected by LC-MS and LAESI-MS.

**Supplemental Figure S2.** [M+H]⁺ observed product ions of anthocyanins, luteolin-derived flavonoids, and apigenin-derived flavonoids that are commonly detected in both LCMS and LAESI-MS analyses of MeOH extract of *Phalaenopsis* spp. petals.

**Supplemental Figure S3.** Isomeric forms of apigenin-derived flavonoids from white and purple regions of *Phalaenopsis* spp. petals resolved by high resolution LC-MS.

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