Review

Plant Single-Cell Metabolomics—Challenges and Perspectives

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Abstract: Omics approaches for investigating biological systems were introduced in the mid-1990s and quickly consolidated to become a fundamental pillar of modern biology. The idea of measuring the whole complement of genes, transcripts, proteins, and metabolites has since become widespread and routinely adopted in the pursuit of an infinity of scientific questions. Incremental improvements over technical aspects such as sampling, sensitivity, cost, and throughput pushed even further the boundaries of what these techniques can achieve. In this context, single-cell genomics and transcriptomics quickly became a well-established tool to answer fundamental questions challenging to assess at a whole tissue level. Following a similar trend as the original development of these techniques, proteomics alternatives for single-cell exploration have become more accessible and reliable, whilst metabolomics lag behind the rest. This review summarizes state-of-the-art technologies for spatially resolved metabolomics analysis, as well as the challenges hindering the achievement of sensu stricto metabolome coverage at the single-cell level. Furthermore, we discuss several essential contributions to understanding plant single-cell metabolism, finishing with our opinion on near-future developments and relevant scientific questions that will hopefully be tackled by incorporating these new exciting technologies.

Keywords: cell type specific metabolism; metabolomics; single-cell; mass spectrometry imaging

1. Introduction

The advent of genomics immediately followed by similar conceptual frameworks to investigate transcriptomes, proteomes, and metabolomes represented a paradigm shift in biological systems investigation. The appealing idea of holistically assessing such systems has translated into rapid developments for systems biology. Researchers can now investigate multiple processes simultaneously, revealing essential mechanisms involved in regulating development and responses to the environment. For practical reasons, such techniques have been mainly applied to bulk samples consisting of a large number of cells for which results correspond to populations’ averages (Figure 1). In such experiments, the stochasticity of biological processes leading to cell heterogeneity is often considered not to be biologically relevant. Indeed, this is often the case, and for many applications such as characterizing mutants of central metabolic pathways [1,2] and identifying genes involved in the production of specialized metabolites [3–5], the use of averages is undoubtedly suitable.

However, cell heterogeneity has been shown to play important biological roles in many situations for which averaging would mask relevant mechanistic insights [6]. In plants, several works highlighted the importance of cell-specific metabolism in regulating essential physiological processes such as the metabolism of the shoot apical meristem [7], the regulation of stomatal closure by guard cells and subsidiary cells [8,9], C4 metabolism [10–12], and the evolution of specialized metabolism [13].
However, most of these studies involve cell-specific labor-intensive protocols for cell isolation or reporter lines targeting few metabolites. True metabolomics at the cellular level remains a daunting task due to innumerable challenges in measuring metabolites.

2. Technical Challenges

Current coverage of the metabolome still lags far behind genomics, transcriptomics, and proteomics because of the technical limitations imposed by the nature of metabolites. DNA, RNA, and proteins exhibit high regularity as they are constituted by a set of repeating unities, namely nucleotides and amino acids. As a consequence, these classes of molecules have characteristic physicochemical properties that are similar between them. Metabolites, on the other hand, exhibit much broader physicochemical diversity hindering their global analysis by a single technique. The broadest coverages of the metabolome achievable by date rely heavily upon the high sensitivity of mass spectrometry techniques hyphenated to efficient separation provided by gas and liquid chromatography. Although current technological advances provide considerable resolution in benchmark equipment such as orbitraps and quadrupole time-of-flight mass spectrometers (QTOFs), the combination of these two techniques is still essential for overcoming matrix effects providing maximum metabolome coverage.

Following the trend, metabolomics once again lags its predecessors in the pursuit of single-cell systems biology. Single-cell genomics and transcriptomics saw rapid popularization in the last years [14], followed more recently by proteomics [15–17]. Here, in addition to the aforementioned technical hurdles, sensitivity also imposes a challenge for single-cell metabolomics. DNA and RNA analysis presents a significant technical advantage as the genetic material can be amplified, yielding considerably more sensitive detection over proteins and metabolites. Recent developments in proteomics have explored alternatives such as fluorescent tags providing a comprehensive increase in sensitivity. On the other hand, metabolites cannot be amplified, and their broad dynamic range of concentrations has a considerable impact on the observable metabolome. Furthermore, improving detection through derivatization reactions is also complicated by their wide chemical diversity and a higher propensity to structural modifications compared to bulkier proteins. Moreover, the minute concentrations and volumes of material represent an issue for using classical platforms relying on chromatographic separation.

We can classify attempts to achieve cellular resolution metabolomics in three main groups: those that attempt at isolating enough material of a specific cell type to perform the analysis on platforms used for regular metabolomics, which we will refer to as single-cell-type metabolomics as coined by Reference [18]; those based on micromanipulation of single cells [19,20]; and those based on...
A more viable alternative from the analytical point of view is to sample many specific cells before the metabolomics experiment in single-cell-type experiments [18]. The main advantage is the possibility of using traditional LC/GC-MS-based platforms providing high throughput, optimal sensitivity, and coverage due to the chromatographic separation. The main limitations are imposed by the chosen cell sampling technique [24–26]. Some particularly exciting works include applications of laser microdissection (LMD)-based techniques such as laser microdissection and pressure catapulting (LMPC) and laser capture microdissection (LCM) [18], as well as fluorescence-activated cell sorting (FACS) [27].

LMD-based techniques are a great option as they preserve contextual information from spatial cell distribution. However, they are significantly limited in terms of throughput. LMD is a labor-intensive technique requiring an experienced operator to harvest the cells [18]. FACS, on the other hand, provides a high throughput alternative to isolating specific cells. However, the necessity to obtain single-cell suspensions is far from trivial, considerably affecting the metabolome [28]. Moreover,
the inherent introduction of perturbations due to cell manipulation by all these techniques is particularly troublesome when considering the rapid changes of the metabolome with the turnover time of some metabolites being fractions of a second [29]. Despite these limitations, the recent improvement in data processing capacity and machine learning algorithms brings exciting advances to fill some of these gaps. A great example has recently been shown using image analysis algorithms, machine-learning, and high-throughput microscopy to recognize individual cells in suspensions or tissue and automatically guide extraction through LCM or micromanipulation in the so-called computer-assisted microscopy isolation (CAMI) [30]. Similarly, exciting improvements have also been developed for FACS [31]. However, the issues related to obtaining cell suspensions for this technique are likely hard to overcome and particularly challenging for plant sciences, as discussed below.

4. Mass Spectrometry Imaging (MSI)

MSI is a general term encompassing multiple technologies capable of providing spatially resolved ionization of samples for mass spectrometry-based metabolite profiling [21,22]. The multiple techniques essentially provide different tradeoffs related to sample preparation, the lateral resolution of the ionization event, degree of fragmentation, and ionization range ($m/z$). We briefly describe here some of the most common ionization platforms that we believe cover an attractive complementary space of features, namely matrix-assisted laser desorption/ionization (MALDI) [32,33], secondary ion mass spectrometry (SIMS) [34], desorption electrospray ionization (DESI) [35,36], and laser-ablation electrospray ionization (LAESI) [37] (Figure 3).

![Figure 3. Schematic representation of the different ionization strategies used for mass spectrometry imaging (MSI). (A) MALDI, (B) secondary ion mass spectrometry (SIMS), (C) desorption electrospray ionization (DESI), (D) laser-ablation electrospray ionization (LAESI).](image)

MALDI is the most popular ionization method adaptable to MSI [38]. In MALDI, a matrix applied to the sample is excited by a laser; this energy is further transferred to the sample resulting in the ionization event [32,33]. It is particularly good at ionizing large molecules above 500 $m/z$, often suffering from matrix interference signals below this mass range [39]. Several groups have developed extensive work involving MALDI’s application as a platform for MSI with multiple applications into the analysis of plant samples [22]. Despite limited biologically relevant insights, these works tackle some of
the main challenges in achieving comprehensive spatially resolved metabolomics, including sample preparation, the lateral resolution of ionization, and multiplex data acquisition.

Preparation for MALDI usually comprises cryo-sectioning and lyophilizing a frozen sample embedded in some media before applying the matrix by either a sprayer or solvent-free sublimation [39]. These methods offer an advantage over cell isolation in terms of metabolome integrity whilst also preserving the relative localization of cells and allowing them to assess the intercellular space [40]. However, the process still lacks significant improvements in throughput. The choice of method for matrix deposition and its composition are particularly important factors in MALDI ionization. Comparing traditional spray and solvent-free sublimation methods as an example show that the former may promote metabolite delocalization, an issue amended by the latter method in the detriment of other metabolites not being ionized [41]. Moreover, matrix crystalline structure is a relevant factor limiting lateral resolution [42]. That said, matrix optimization is an active field in technological developments for MALDI imaging applications [43]. Several works have described matrix optimization for specific compound classes [44], as well as exciting approaches to expand the coverage based on derivatizations [45] and post-ionization strategies [46]. Another recent trend involves using nanoparticles instead of organic matrices, and it shows promising results for ionizing the smaller range of metabolites and providing increased spatial resolution [47,48].

MALDI’s lateral resolution is usually in the range of 50–10 μm, even though some reports manage to achieve numbers as low as 2–5 μm in customized systems [40,49]. Factors limiting resolution again include the matrix structure and also qualitative aspects of the laser. UV lasers provide higher resolutions of up to 10 μm. However, they have several disadvantages compared to IR lasers, such as limitations in matrix absorption [21]. SIMS is an alternative to MALDI that relies on ion beams instead of a laser to ionize the samples [50]. Such a mechanism results in a more fragmented ionization and removes the necessity of any matrix and limitations due to laser’s diffraction limit, thus providing higher reproducibility and resolution below 2 μm [50]. Moreover, SIMS allows for the acquisition of 3D imaging through the use of dual beams. Indeed, all these advantages have been recently combined in a commercial system that includes the ultra-high resolution of orbitrap analyzers [51].

Despite the advantage of more straightforward sample preparation, SIMS-based platforms’ limitation is the need for samples to be ionized under a high vacuum. A few works try overcoming such limitations, for instance, via the use of cryogenic orbiSIMS to evaluate semi-volatile organic compounds that would otherwise be vaporized before ionization [52]. Nevertheless, DESI and LAESI offer promising alternatives for direct ionization of samples with minimal treatment. In DESI, a solvent stream originated from an electrospray probe is directed at an angle toward the sample at ambient pressure, propelling secondary ions to the analyzer [53]. One of the biggest limitations of DESI is its comparatively low resolution in the order of 100 μm [50]. Finally, LAESI combines laser ablation followed by post-ionization via an electrospray. A typical resolution is in the order of 200–300 μm; however, it can reach better resolution than DESI with the additional advantage of ionizing through multiple layers of tissue [21,54–56].

Despite its many advantages concerning in situ analysis, MSI platforms offer significant challenges regarding data analysis [57]. As a technique in its infancy, data processing standards, such as normalization, are still lacking. Indeed, only relatively recently, an open cross-platform data format was developed [58]. Quantification is also challenging, with few works providing absolute metabolite levels [59].

5. Spatially Resolved Metabolomics in Plants: Current Status, Challenges, and Future Prospects

The tremendous metabolic diversity that evolved in plants at the level of tissues and organs makes single-cell metabolomics a suitable tool for investigations targeting these cell-specific chemical signatures.

To resolve metabolic diversity at a tissue level, spatial single-cell mass spectrometry, performed alone or in combination with single-cell mass spectrometry, has primarily been applied to plant science
In *Catharanthus roseus*, for example, the combination of MSI and single-cell MS provided evidence of a developmentally driven process that segregates branches of the terpenoid indole alkaloid (TIA) biosynthetic pathway into specific anatomical structures. As idioblasts and laticifers differentiate while leaves grow and expand, single-cell MS detected the appearance of new metabolic intermediates so that an initial draft of the TIA pathway could be written in its completeness once leaves fully developed [60,61]. From an evolutionary perspective, this is a remarkable discovery as it provides an additional example of the parallel evolution between biochemical processes and anatomical structures, which often occurs in plants. In a similarly elegant experiment, Livingston and colleagues used a combination of different techniques, which included measurements of trichome intrinsic fluorescence and microcapillary-assisted metabolite extraction followed by GC-MS, UHPLC-MS/MS, and RNA sequencing analyses, to lay out the developmental trajectories of *Cannabis sativa* trichomes from sessile to stalked and the parallel changes occurring in the composition of their metabolites [62].

Questions concerning color pattern formation in flowers have also been answered via MSI. For instance, a recent study revealed that the deep-blue color of the nectar guides of *Viola cornuta* petals is due to the colocalization of the anthocyanin violanin and numerous colorless flavonol 3-O-glycosides [63]. By surrounding violanin, flavonol molecules prevent self-stacking and the consequent shift in the spectrum of light absorbance [64]. Additionally, they protect the chromophore of violanin from hydration, hence inhibiting the formation of colorless chalcones.

It is not accidental that the great majority of these studies focused on tissues that accumulate specialized metabolites in a large abundance and can be relatively easily accessed, such as glandular trichomes [62,65–67], laticifers [60,68], and floral petals [69–71]. Indeed, as metabolites physiologically accumulate in these organs and structures, their concentration is already optimized to detect an MS signal of sufficient quality for the molecular identification of compounds. In all other cases, to achieve a proper concentration, metabolites must be extracted from a hundred thousand identical cells. For reasons that we explain below, harvesting such a large number of cells from plant tissues is extremely labor-intensive, as it emerges when comparisons with similar systems utilized in animal studies are made.

Animal cell lines established after cell disaggregation from tissues followed by subculturing usually maintain similar physiological and biochemical characteristics as their organ of origin [72]. As such, immortal animal cell lines, for example, HeLa cells, have been successfully utilized to investigate metabolic responses to drugs and growth regulators [57]. Indeed, as metabolic changes in animal cell cultures mirror changes in intact organs, single-cell metabolomics is a powerful system to predict metabolic trajectories induced by medical treatments [73]. Conversely, plant liquid and solid (callus) cultures are made of cells in an undifferentiated status maintained with a balanced ratio of auxins and cytokinins [74]. As plant cell cultures are phenotypically and biochemically very distant from their differentiated counterparts, methods other than culturing must be adopted to collect a large number of cells of a specific lineage. Fluorescence-activated cell sorting (FACS) has been successfully employed to collect a large amount of GFP-tagged lines from plant tissues [27], but FACS applied to samples destined for the analysis of metabolites is a very challenging procedure. As reagents for cell protoplasting are potential contaminants of the MS detector and metabolites are prone to fast degradation, well-established methods that are used to collect RNA from fluorescent-tagged and sorted cells need further adjustments when applied to single-cell metabolomics [27]. In addition, the spatial distribution of differentiated cells in plant tissues and the prospect of obtaining protoplasts from these cells are tremendous limitations to the pursuit of harvesting cells of a single type in an amount that is sufficient for metabolite analysis. In roots, the continuous development and radial organization of layered tissues make single-cell collection via FACS relatively easily attainable, as well as facilitating the interpretation of imaging at a reasonable lateral resolution [75–77]. Conversely, organs that at maturity show a high degree of anatomical complexity, for example, flowers, are not equally suitable for such analyses. Not to mention that the process of protoplasting, which removes the cell wall—an intrinsic component of all plant cells—washes away compounds that cells secrete and deposit in
the extracellular space. These compounds often have relevant physiological functions, for example, phenylalanine derivatives which confer protection to fungal pathogens [78]. Therefore, the removal of the cell wall can make data interpretation difficult, as it may weaken the link between chemical phenotypes and physiological functions.

These challenges are at least partially resolved when tissues undergoing single-cell analyses are already composed of a large number of identical cells. Thus, for seeds and grains where cells with well-defined chemistry spatially cluster to form seed coat, embryo, and endosperm, MSI has largely been utilized to resolve in situ localization of metabolites. For example, in the oil-seed crops *Camelina sativa* and *Brassica napus*, as well as in Arabidopsis, MSI helped to determine the distribution of lipids in the embryo of wild-type and transgenic lines [79–83]. In barley and wheat, the spatial distribution of sugars and proteins between the endosperm and aleurone layer has been the main object of study [84–86]. Furthermore, in inbreds of maize, amino acids, sugar alcohols, organic acids, phospholipids, and triacylglycerols were observed within the embryo and radicle [87]. Unicellular structures and unicellular organisms such as pollen grains, algae, and microalgae (diatoms) represent another exception. For instance, metabolomics of pollen grains, which are unicellular haploid male gametophytes, has been performed with the most disparate array of techniques. As a result of these investigations, the molecular structure and composition of sporopollenin have recently been unraveled [88,89]. Sporopollenin is an extraordinarily inert and resistant polymer, the acquisition of which by land plants represents a focal adaptation to life outside water. The spectacular inertness of sporopollenin toward the most disparate analytical techniques made the search for its molecular structure hard to obtain, and at the same time, very desirable given the multitude of promising applications in the fields of material engineering and nanotechnology. Progress has also been made in the analysis of lipids, proteins, and the mechanisms of accumulation of flavonoid glycosides on the surface of pollen grains [90–93], as well as the metabolic processes underlying pollen germination and pollen tube elongation [94]. However, given the complexity of these chemical signals, their physiological function is not yet fully understood [95]. Finally, we briefly mention here that while collecting abundant pollen from male microsporangia of gymnosperms is usually easy endeavors, autogamous angiosperms generally produce a tiny amount of pollen, for which the collection of whole anthers is a necessary step.

In algal research, live single-cell metabolomics helped elucidate the metabolic rearrangements occurring in response to environmental perturbations such as low nutrient and variation in light regimes [96–98]. Phytoplankton, which is primarily composed of microalgae and minor amounts of protists and bacteria, contributes to global biogeochemical cycles of carbon, nitrogen, phosphorus, and silicate. Therefore, gaining an understanding of the physiological status of phytoplankton cells holds excellent promises for environmental research. Besides, chemotyping of microalgae via pipelines that utilize live single-cell MS is currently exploited for taxonomic identification [99]. Despite the broad applications in environmental research, initial studies on algal metabolomics mostly focused on the model organism *Chlamydomonas reinhardtii* because of applications in the biotechnology industry and biofuel production [100]. In the recent past, metabolomic and transcriptomic approaches have been extensively utilized to investigate the responses to external determinants of algal growth such as temperature, light intensity, salinity, and nutrient availability [101,102], while today’s research mostly shifted toward functional genomic studies that aim at understanding the genetic mechanisms of this metabolic plasticity [103].

In plants, the application of single-cell MS to functional genomic studies has so far been scant, although initial studies that employed known *Arabidopsis thaliana* mutants as a proof of concept showed great promises [104–106]. More recently, the combination of direct infusion metabolomics and MSI was used to characterize the signaling pathway of feronia mutants, revealing an interesting phenotype associated with high levels of oxylipin arbidopsides, and suggesting chloroplastic localization [107]. Other new prospects of single-cell metabolomic applications pertain to research on plant–pathogen interactions where MSI is currently being utilized to analyze plant metabolites synthesized in response to pathogens’ infection. Here, initial studies that used to visualize metabolites present on the plant
surface have further expanded to include analysis of metabolites that accumulate deeper in plant tissues, which can be seen after tissue fracturing and sectioning \[108\]. A recent study has shown this new approach’s relevance when it analyzed transcriptome and metabolome responses of susceptible and resistant soybean cultivars to aphid infestation. As aphids are insects that feed on phloem sap, plant metabolites conferring resistance to aphids are expected to be found in the phloem. Conversely, MSI revealed the accumulation of isoflavones in mesophyll and epidermal cells, suggesting a role for these compounds in the non-phloem defense response induced by feeding \[109\]. MSI has also been used to investigate the distribution of glucosinolates across Arabidopsis leaves and the response of lepidopterans oviposition to the detected metabolites’ concentrations \[59\]. In plant–bacteria symbiotic associations, MSI has been employed to study metabolite distribution in roots and nodules of wild-type and mutant genotypes of *Medicago truncatula* \[110,111\], and more recently of soybean \[112,113\].

Still, single-cell metabolomics has found very little application in the field of plant developmental research where the combination of functional genomics and metabolomics holds the promise to pave the way toward a better understanding of how and to what extent anatomy and metabolism are mutually coordinated. This is at least partially due to challenges related to sample preparation, as the presence of abundant water, cell wall, and cuticles make the process laborious in plants \[114\]. Additionally, the resolution and annotation of metabolites of the central pathway, which accumulate in lower abundance than specialized metabolites (see above), represent an additional challenge. Similarly, phytohormones that play crucial roles at the cellular level in the development and environmental responses are of great interest but challenging to detect even with traditional methods \[115\]. A work using the “Live-MS” platform \[116\] to investigate the response of two phytohormones, ABA and JA-Ile, have shown promising results being able to detect some of the expected changes. However, their results also point to limitations of the technique which still suffers from high variability. Finally, it is worth mentioning that plants primarily utilize hexoses and various sugar polymers for storage, transport, and organ to organ communication, of which MS annotation is not always easily attainable.
Table 1. Summary of spatially resolved plant metabolomics works applying mass spectrometry-based platforms described in this review.

| Species          | Technique       | Cell-Type/Tissue                                                                 | Compounds                        | Reference |
|------------------|-----------------|----------------------------------------------------------------------------------|----------------------------------|-----------|
| Arabidopsis      | FACS            | Roots                                                                            | Multiple                          | [27]      |
| Arabidopsis      | MALDI           | Leaves                                                                           | Glucosinolates                   | [60]      |
| Catharanthus roseus | MALDI and Live-MS | Laticifers and idioblasts from leaves                                           | TIA                              | [61]      |
| Catharanthus roseus | MALDI and Live-MS | Laticifers, idioblast, parenchyma, and epidermal cells from stems               | TIA                              | [62]      |
| Viola cornuta    | MALDI           | Petals                                                                           | Flavonoids                       | [64]      |
| Rauvolfia tetraphylla | DESI             | Stem, leaves, root, and fruits                                                   | Indole alkaloids                 | [69]      |
| Hypericum perforatum | DESI            | Petals and leaves                                                                | Hyperforin                        | [71]      |
| Datura stramonium | DESI            | Petals and leaves                                                                | Sugars, atropine, and scopolamine | [71]      |
| Maize            | MALDI           | Roots                                                                            | Amino acids                      | [76]      |
| Maize            | MALDI           | Roots                                                                            | Lipids, sugars, and benzoazinoid  | [77]      |
| Glycyrhriza glabra | MALDI           | Roots                                                                            | Flavonoids and triterpenoids     | [78]      |
| Camelina sativa  | MALDI           | Seed                                                                             | Lipids                           | [80]      |
| Camelina sativa  | MALDI           | Seed                                                                             | Lipids                           | [81]      |
| Camelina sativa  | MALDI           | Seed                                                                             | Lipids                           | [82]      |
| Brassica napus   | MALDI           | Seed                                                                             | Lipids                           | [83]      |
| Arabidopsis      | MALDI           | Seed                                                                             | Lipids                           | [84]      |
| Barley           | MALDI           | Germinating seeds                                                               | Multiple                          | [85]      |
| Maize            | MALDI           | Germinating seeds                                                               | Multiple                          | [88]      |
| Lycopodium clavatum | SIMS and MALDI | Polen                                                                            | Sporopollenin                    | [90]      |
| Poa alpina       | MALDI           | Polen                                                                            | Multiple                          | [91]      |
| Arabidopsis      | MALDI           | Leaves                                                                           | Oxyllipins                        | [108]     |
| Rice             | MALDI           | Leaves                                                                           | Multiple                          | [109]     |
| Soybean          | MALDI           | Leaves                                                                           | Multiple                          | [109]     |
| Soybean          | MALDI           | Leaves                                                                           | Isolavones                       | [110]     |
| Medicago truncatula | MALDI          | Root nodules                                                                     | Multiple                          | [111]     |
| Medicago truncatula | MALDI          | Root nodules                                                                     | Multiple                          | [112]     |
| Soybean          | MALDI           | Root nodules                                                                     | Multiple                          | [113]     |
| Soybean          | LAESI           | Root nodules                                                                     | Multiple                          | [114]     |
| Vicia faba       | Live-MS         | Leaves                                                                           | Phytohormones                    | [117]     |
6. Conclusions and Future Perspectives

There are just over 40 different types of cells described in plant tissues [117]. As most metabolomics experiments capture data of whole tissues, our knowledge is largely biased toward prevailing cells such as mesophyll cells in leaves [118] and endosperm in seeds [119,120]. However, several works highlight the striking differences in cell-specific metabolism and the impact that less recurrent cell types have in regulating and integrating crucial physiological processes, including transpiration and photosynthesis [121,122]. Moreover, assessing metabolic heterogeneity across cells belonging to a tissue has the potential to unravel unforeseen details masked by averaging such populations of cells, thereby contributing to a deeper understanding of metabolic regulation [6].

Techniques for measuring single-cell metabolites have recently gone through considerable improvements providing exciting insights into metabolic compartmentalization. Nevertheless, some of the metabolomics most outstanding achievements rely on high throughput and comprehensive metabolome coverage. Both parameters are still considerably limited in the current single-cell and spatially resolved platforms. The many advantages of single-cell profiling described here represent an enormous potential when applied to large throughput experiments. Single-cell transcriptomics of different human tissues has recently been utilized to identify Quantitative Trait Loci (QTL) associated with expression and splicing variants (eQTLs and sQTLs, respectively) affected by the background genetic variation of different individuals [123]. A similar approach to plant tissues has not yet been adopted. However, it represents a significant potential if applied to large populations to understand, among others, the effect of environmental perturbations at a single-cell level.

Improvements in various aspects of mass spectrometry aspects, particularly resolution and sensitivity, have been instrumental in facilitating the measurement of the spatial distribution of metabolites through single-cell and MSI platforms. The introduction and broad adoption of other technologies into metabolomics platforms, such as nanoLC and ion mobility, are likely to play important roles in further reducing issues concerning limited sample and sensitivity in single-cell metabolomics and matrix effects in MSI, respectively. Better integration of current technologies with other imaging platforms such as microscopy also offers a promising way to improve experiments throughput and information [73].

Finally, as these technologies mature, we can foresee their adoption to even the most challenging applications in current tissue level metabolomics. A recurrent question that has proven essential to characterize metabolism is the definition of metabolic fluxes rather than a simple description of relative metabolite levels as routinely performed [124]. Methods for integrating multi-omics of single cells are also an exciting boundary to be crossed [125]. We can anticipate considerable hurdles for generating such datasets. However, this could represent an outstanding means of reducing experimental complexity while improving the statistical power of systems biology studies.

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Abbreviations

DESI Desorption electrospray ionization
FACS Fluorescence-activated cell sorting
GC Gas chromatography
LAESI Laser-ablation electrospray ionization
LC Liquid chromatography
LCM Laser capture microdissection
LMD Laser microdissection
References

1. Daloso, D.M.; Müller, K.; Obata, T.; Florian, A.; Tohge, T.; Bottcher, A.; Riondet, C.; Bariat, L.; Carrari, F.; Nunes-Nesi, A.; et al. Thioredoxin, a master regulator of the tricarboxylic acid cycle in plant mitochondria. Proc. Natl. Acad. Sci. USA 2015, 112, E1392–E1400. [CrossRef] [PubMed]

2. Araújo, W.L.; Ishizaki, K.; Nunes-Nesi, A.; Larson, T.R.; Tohge, T.; Krahnert, I.; Witt, S.; Obata, T.; Schauer, N.; Graham, I.A.; et al. Identification of the 2-Hydroxyglutarate and Isovaleryl-CoA Dehydrogenases as Alternative Electron Donors Linking Lysine Catabolism to the Electron Transport Chain of Arabidopsis Mitochondria. Plant Cell 2010, 22, 1549–1563. [CrossRef] [PubMed]

3. Tohge, T.; Wendenburg, R.; Ishihara, H.; Nakabayashi, R.; Watanabe, M.; Sulpice, R.; Hoefgen, R.; Takayama, H.; Saito, K.; Stitt, M.; et al. Characterization of a recently evolved flavonol-phenylacyltransferase gene provides signatures of natural light selection in Brassicaceae. Nat. Commun. 2016, 7, 12399. [CrossRef] [PubMed]

4. Perez de Souza, L.; Garbowicz, K.; Brotman, Y.; Tohge, T.; Fernie, A.R. The Acetate Pathway Supports Flavonoid and Lipid Biosynthesis in Arabidopsis. Plant Physiol. 2020, 182, 857–869. [CrossRef] [PubMed]

5. Cárcedas, P.D.; Sonawane, P.D.; Heinig, U.; Iozziak, A.; Panda, S.; Abebie, B.; Kazachkova, Y.; Pliner, M.; Unger, T.; Wolf, D.; et al. Pathways to defense metabolites and evading fruit bitterness in genus Solanum evolved through 2-oxoglutarate-dependent dioxygenases. Nat. Commun. 2019, 10, 5169. [CrossRef] [PubMed]

6. Altschuler, S.J.; Wu, L.F. Cellular Heterogeneity: Do Differences Make a Difference? Cell 2010, 141, 559–563. [CrossRef]

7. Fleming, A. Metabolic aspects of organogenesis in the shoot apical meristem. J. Exp. Bot. 2006, 57, 1863–1870. [CrossRef]

8. Medeiros, D.B.; Perez Souza, L.; Antunes, W.C.; Araújo, W.L.; Daloso, D.M.; Fernie, A.R. Sucrose breakdown within guard cells provides substrates for glycolysis and glutamine biosynthesis during light-induced stomatal opening. Plant J. 2018, 94, 583–594. [CrossRef]

9. Wang, H.; Yan, S.; Xin, H.; Huang, W.; Zhang, H.; Teng, S.; Yu, Y.-C.; Fernie, A.R.; Lu, X.; Li, P.; et al. A Subsidiary Cell-Localized Glucose Transporter Promotes Stomatal Conductance and Photosynthesis. Plant Cell 2019, 31, 1328–1343. [CrossRef]

10. Arrivault, S.; Obata, T.; Szecówka, M.; Mengin, V.; Guenther, M.; Hoehne, M.; Fernie, A.R.; Stitt, M. Metabolite pools and carbon flow during C4 photosynthesis in maize: 13CO2 labeling kinetics and cell type fractionation. J. Exp. Bot. 2016, 68, 283–298. [CrossRef]

11. Islam, M.M.; Al-Siyabi, A.; Saha, R.; Obata, T. Dissecting metabolic flux in C4 plants: Experimental and theoretical approaches. Phytochem. Rev. 2018, 17, 1253–1274. [CrossRef]

12. Weissmann, S.; Ma, F.; Furuyama, K.; Gierse, J.; Berg, H.; Shao, Y.; Taniguchi, M.; Allen, D.K.; Brutnell, T.P. Interactions of C4 Subtype Metabolic Activities and Transport in Maize Are Revealed through the Characterization of DCT2 Mutants. Plant Cell 2016, 28, 466–484. [CrossRef] [PubMed]

13. Schenck, C.A.; Last, R.L. Location, location! cellular relocalization primes specialized metabolic diversification. FEBS J. 2020, 287, 1359–1368. [CrossRef]

14. Slavov, N. Unpicking the proteome in single cells. Science 2020, 367, 512–513. [CrossRef]
18. Misra, B.B.; Assmann, S.M.; Chen, S. Plant single-cell and single-cell-type metabolomics. *Trends Plant Sci.* **2014**, *19*, 637–646. [CrossRef]

19. Fuji, T.; Matsuoka, S.; Tejedor, M.L.; Esaki, T.; Sakane, I.; Mizuno, H.; Tsuyama, N.; Masujima, T. Direct metabolomics for plant cells by live single-cell mass spectrometry. *Nat. Protoc.* **2015**, *10*, 1445–1456. [CrossRef]

20. Yang, Y.; Huang, Y.; Wu, J.; Liu, N.; Deng, J.; Luan, T. Single-cell analysis by ambient mass spectrometry. *TrAC Trends Anal. Chem.* **2017**, *90*, 14–26. [CrossRef]

21. Bjarnholt, N.; Li, B.; D’Alvis, J.; Janfelt, C. Mass spectrometry imaging of plant metabolites–principles and possibilities. *Nat. Prod. Rep.* **2014**, *31*, 818–837. [CrossRef] [PubMed]

22. Hansen, R.L.; Lee, Y.J. High-Spatial Resolution Mass Spectrometry Imaging: Toward Single Cell Metabolomics in Plant Tissues. *Chem. Rev.* **2018**, *18*, 65–77. [CrossRef] [PubMed]

23. Masujima, T. Live Single-cell Mass Spectrometry. *Anal. Sci.* **2017**, *33*, 953–960. [CrossRef] [PubMed]

24. Gross, A.; Schoendube, J.; Zimmermann, S.; Steeb, M.; Zengerle, R.; Koltay, P. Technologies for Single-Cell Isolation. *Int. J. Mol. Sci.* **2015**, *16*, 16897–16919. [CrossRef]

25. Zhang, X.; Wei, X.; Wei, Y.; Chen, M.; Wang, J. The up-to-date strategies for the isolation and manipulation of single cells. *Talanta* **2020**, *218*, 121147. [CrossRef]

26. Couvillion, S.P.; Zhu, Y.; Nagy, G.; Adkins, J.N.; Ansong, C.; Renslow, R.S.; Piehowski, P.D.; Ibrahim, Y.M.; Kelly, R.T.; Metz, T.O. New mass spectrometry technologies contributing towards comprehensive and high throughputomics analyses of single cells. *Analyst* **2019**, *144*, 794–807. [CrossRef]

27. Moussaieff, A.; Rogachev, I.; Brodsky, L.; Malitsky, S.; Toal, T.W.; Belcher, H.; Yativ, M.; Brady, S.M.; Benfey, P.N.; Aharoni, A. High-resolution metabolic mapping of cell types in plant roots. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E1232–E1241. [CrossRef] [PubMed]

28. Reichard, A.; Asosingh, K. Best Practices for Preparing a Single Cell Suspension from Solid Tissues for Flow Cytometry. *Cytom. Part A* **2019**, *95*, 219–226. [CrossRef]

29. Arrivault, S.; Guenther, M.; Ivakov, A.; Feil, R.; Vosloh, D.; Van Dongen, J.T.; Sulpice, R.; Stitt, M. Use of...
59. Shroff, R.; Schramm, K.; Jeschke, V.; Nemes, P.; Vertes, A.; Gershenzon, J.; Svatoš, A. Quantification of plant surface metabolites by matrix-assisted laser desorption–ionization mass spectrometry imaging: Glucosinolates on Arabidopsis thaliana leaves. Plant J. 2015, 81, 961–972. [CrossRef]

58. Schramm, T.; Hester, Z.; Klinkert, I.; Both, J.-P.; Heeren, R.M.A.; Brunelle, A.; Lapréevote, O.; Desbenoit, N.; Robbe, M.-F.; Stoeckli, M.; et al. imzML—A common data format for the flexible exchange and processing of mass spectrometry imaging data. Anal. Bioanal. Chem. 2015, 407, 2115–2123. [CrossRef] [PubMed]

57. Alexandrov, T. Spatial Metabolomics and Imaging Mass Spectrometry in the Age of Artificial Intelligence. Annu. Rev. Biomed. Data Sci. 2020, 3, 61–87. [CrossRef]

56. Samarah, L.Z.; Khattar, R.; Tran, T.H.; Stopka, S.A.; Brantner, C.A.; Parlanti, P.; Velićković, D.; Shaw, J.B.; Schramm, T.; Hester, Z.; Klinkert, I.; Both, J.-P.; Heeren, R.M.A.; Brunelle, A.; Lapréevote, O.; Desbenoit, N.; Robbe, M.-F.; Stoeckli, M.; et al. imzML—A common data format for the flexible exchange and processing of mass spectrometry imaging data. J. Proteom. 2012, 75, 5106–5110. [CrossRef] [PubMed]

55. Samarah, L.Z.; Khattar, R.; Tran, T.H.; Stopka, S.A.; Brantner, C.A.; Parlanti, P.; Velićković, D.; Shaw, J.B.; Schramm, T.; Hester, Z.; Klinkert, I.; Both, J.-P.; Heeren, R.M.A.; Brunelle, A.; Lapréevote, O.; Desbenoit, N.; Robbe, M.-F.; Stoeckli, M.; et al. imzML—A common data format for the flexible exchange and processing of mass spectrometry imaging data. J. Proteom. 2012, 75, 5106–5110. [CrossRef] [PubMed]

54. Kulkarni, P.; Wilschut, R.A.; Verhoeven, K.J.F.; van der Putten, W.H.; Garbeva, P. LAESI mass spectrometry imaging as a tool to differentiate the root metabolome of native and range-expanding plant species. Plant Physiol. 2015, 169, 1424–1435. [CrossRef] [PubMed]

53. Wiseman, J.M.; Ifa, D.R.; Song, Q.; Cooks, R.G. Tissue Imaging at Atmospheric Pressure Using Desorption Electrospray Ionization (DESI) Mass Spectrometry. Angew. Chem. Int. Ed. 2006, 45, 7188–7192. [CrossRef] [PubMed]

52. Newell, C.L.; Vorng, J.-L.; MacRae, J.I.; Gilmore, I.S.; Gould, A.P. Cryogenic OrbiSIMS Localizes Semi-Volatile Metabolites in Biological Tissues. Angew. Chem. Int. Ed. 2020, 59, 18194–18200. [CrossRef] [PubMed]

51. Passarelli, M.K.; Pirkl, A.; Moellers, R.; Grinfeld, D.; Kollmer, F.; Marshall, P.S.; Arlinghaus, H.; Alexander, M.R.; et al. The 3D OrbiSIMS—Label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. Nat. Methods 2017, 14, 1175–1183. [CrossRef] [PubMed]

50. Touboul, D.; Brunelle, A. What more can TOF-SIMS bring than other MS imaging methods? Bioanalysis 2016, 8, 367–369. [CrossRef] [PubMed]

49. Dueñas, M.E.; Larson, E.A.; Lee, Y.J. Toward Mass Spectrometry Imaging in the Metabolomics Scale: Increasing Metabolic Coverage Through Multiple On-Tissue Chemical Modifications. Front. Plant Sci. 2019, 10. [CrossRef]

48. Hansen, R.L.; Dueñas, M.E.; Lee, Y.J. Sputter-Coated Metal Screening for Small Molecule Analysis and High-Spatial Resolution Imaging in Laser Desorption Ionization Mass Spectrometry. J. Am. Soc. Mass Spectrom. 2019, 30, 299–308. [CrossRef] [PubMed]

47. Yagnik, G.B.; Hansen, R.L.; Korte, A.R.; Reichert, M.D.; Vela, J.; Lee, Y.J. Large Scale Nanoparticle Screening for Subcellular-level resolution MALDI-MS imaging and imaging of small molecule metabolites with 1,5-diaminonaphthalene (DAN). J. Mass Spectrom. 2014, 49, 737–741. [CrossRef] [PubMed]

46. Soltwisch, J.; Kettling, H.; Vens-Cappell, S.; Wiegelmann, M.; Mühling, J.; Dreisewerd, K. Mass spectrometry imaging with laser-induced postionization. Science 2015, 348, 211–215. [CrossRef] [PubMed]

45. Dueñas, M.E.; Larson, E.A.; Lee, Y.J. Toward Mass Spectrometry Imaging in the Metabolomics Scale: Increasing Metabolic Coverage Through Multiple On-Tissue Chemical Modifications. Front. Plant Sci. 2019, 10. [CrossRef]

44. Korte, A.R.; Lee, Y.J. MALDI-MS analysis and imaging of small molecule metabolites with 1,5-diaminonaphthalene (DAN). J. Mass Spectrom. 2014, 49, 737–741. [CrossRef] [PubMed]

43. Calvano, C.D.; Monopoli, A.; Cataldi, T.R.I.; Palmisano, F. MALDI matrices for low molecular weight compounds: An endless story? Anal. Bioanal. Chem. 2018, 410, 4015–4038. [CrossRef] [PubMed]

42. Li, S.; Zhang, Y.; Liu, J.; Han, J.; Guan, M.; Yang, H.; Lin, Y.; Xiong, S.; Zhao, Z. Electrospray deposition of plant surface metabolites by matrix-assisted laser desorption–ionization mass spectrometry imaging: Agtuca, B.J.; Stacey, G.; et al. Single-Cell Metabolic Profiling: Metabolite Formulas from Isotopic Fine Structures in Heterogeneous Plant Cell Populations. Anal. Chem. 2020, 92, 7289–7298. [CrossRef] [PubMed]

41. Gemperline, E.; Rawson, S.; Li, L. Optimization and Comparison of Multiple MALDI Matrix Application Methods for Small Molecule Mass Spectrometric Imaging. Anal. Chem. 2014, 86, 10030–10035. [CrossRef]

40. Korte, A.R.; Yandeau-Nelson, M.D.; Nikolau, B.J.; Lee, Y.J. Subcellular-level resolution MALDI-MS imaging of maize leaf metabolites by MALDI-linear ion trap-Orbitrap mass spectrometer. Anal. Bioanal. Chem. 2015, 407, 2301–2309. [CrossRef] [PubMed]
60. Yamamoto, K.; Takahashi, K.; Caputi, L.; Mizuno, H.; Rodriguez-Lopez, C.E.; Iwasaki, T.; Ishizaki, K.; Fukaki, H.; Ohnishi, M.; Yamazaki, M.; et al. The complexity of intercellular localisation of alkaloids revealed by single-cell metabolomics. New Phytol. 2019, 224, 488–859. [CrossRef]

61. Yamamoto, K.; Takahashi, K.; Mizuno, H.; Anegawa, A.; Ishizaki, K.; Fukaki, H.; Ohnishi, M.; Yamazaki, M.; Masujima, T.; Mimura, T. Cell-specific localisation of alkaloids in Catharanthus roseus stem tissue measured with Imaging MS and Single-cell MS. Proc. Natl. Acad. Sci. USA 2016, 113, 3891–3896. [CrossRef]

62. Livingston, S.J.; Quilichini, T.D.; Booth, J.K.; Wong, D.C.J.; Rensing, K.H.; Lafemme-Yonkman, J.; Castellarin, S.D.; Bohlmann, J.; Page, J.E.; Samuels, A.L. Cannabis glandular trichomes alter morphology and metabolite content during flower maturation. Plant J. 2020, 101, 37–56. [CrossRef]

63. Sugahara, K.; Kitao, K.; Watanabe, T.; Yamagaki, T. Imaging Mass Spectrometry Analysis of Flavonoids in Blue Viola Petals and Their Enclosure Effects on Violanin during Color Expression. Anal. Chem. 2019, 91, 896–902. [CrossRef]

64. Goto, T.; Kondo, T. Structure and Molecular Stacking of Anthocyanins—Flower Color Variation. Angew. Chem. Int. Ed. 1991, 30, 17–33. [CrossRef]

65. Bertea, C.M.; Freije, J.R.; van der Woude, H.; Verstappen, F.W.A.; Perk, L.; Marquez, V.; De Kraker, J.W.; Posthumus, M.A.; Jansen, B.J.M.; de Groot, A.; et al. Identification of Intermediates and Enzymes Involved in the Early Steps of Artemesinin Biosynthesis in Artemisia annua. Planta Med. 2005, 71, 40–47. [CrossRef] [PubMed]

66. Frerigmann, H.; Böttcher, C.; Baatout, D.; Gigolashvili, T. Glucosinolates are produced in trichomes of Arabidopsis thaliana. Front. Plant Sci. 2013, 2. [CrossRef] [PubMed]

67. Schilmiller, A.; Shi, F.; Kim, J.; Charbonneau, A.L.; Holmes, D.; Daniel Jones, A.; Last, R.L. Mass spectrometry screening reveals widespread diversity in trichome specialized metabolites of tomato chromosomal substitution lines. Plant J. 2010, 62, 391–403. [CrossRef] [PubMed]

68. Mohana Kumara, P.; Uma Shaanker, R.; Pradeep, T. UPLC and ESI-MS analysis of metabolites of Rauvolfia tetraphylla L. and their spatial localization using desorption electrospray ionization (DESI) mass spectrometric imaging. Phytochemistry 2019, 159, 20–29. [CrossRef] [PubMed]

69. Shi, F.; Flanigan, P.M.; Archer, J.J.; Levis, R.J. Ambient Molecular Analysis of Biological Tissue Using Low-Energy, Femtosecond Laser Vaporization and Nanospray Postionization Mass Spectrometry. J. Am. Soc. Mass Spectrom. 2016, 27, 542–551. [CrossRef]

70. Thunig, J.; Hansen, S.H.; Janfelt, C. Analysis of Secondary Plant Metabolites by Indirect Desorption Electrospray Ionization Mass Spectrometry. Anal. Chem. 2011, 83, 3256–3259. [CrossRef]

71. Holscher, D.; Shroff, R.; Knop, K.; Gottschaldt, M.; Creccelius, A.; Schneider, B.; Heckel, D.G.; Schubert, U.S.; Svatoš, A. Matrix-free UV-laser desorption/ionization (LDI) mass spectrometric imaging at the single-cell level: Distribution of secondary metabolites of Arabidopsis thaliana and Hypericum species. Plant J. 2009, 60, 907–918. [CrossRef]

72. Bhatia, S.; Naved, T.; Sardana, S. Introduction to Pharmaceutical Biotechnology, Volume 3. In Animal Tissue Culture and Biopharmaceuticals; IOP Publishing: Bristol, UK, 2019.

73. Rappez, L.; Studler, M.; Triana, S.; Phapale, P.; Heikenwalder, M.; Alexandrov, T. Spatial single-cell profiling of intracellular metabolomes in situ. bioRxiv 2019, 510222. [CrossRef]

74. Fehér, A. Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? Front. Plant Sci. 2019, 10. [CrossRef]

75. O’Neill, K.C.; Lee, Y.J. Visualizing Genotypic and Developmental Differences of Free Amino Acids in Maize Roots With Mass Spectrometry Imaging. Front. Plant Sci. 2020, 11. [CrossRef]

76. Feenstra, A.D.; Dueñas, M.E.; Lee, Y.J. Five Micron High Resolution MALDI Mass Spectrometry Imaging with Simple, Interchangeable, Multi-Resolution Optical System. J. Am. Soc. Mass Spectrom. 2017, 28, 434–442. [CrossRef] [PubMed]

77. Li, B.; Bhandari, D.R.; Janfelt, C.; Römpp, A.; Spengler, B. Natural products in Glycyrrhiza glabra (licorice) rhizome imaged at the cellular level by atmospheric pressure matrix-assisted laser desorption/ionization tandem mass spectrometry imaging. Plant J. 2014, 80, 161–171. [CrossRef] [PubMed]

78. Doppler, M.; Kluger, B.; Bueschl, C.; Steiner, B.; Buerstmayr, H.; Lemmens, M.; Kraska, R.; Adam, G.; Schuhmacher, R. Stable Isotope-Assisted Plant Metabolomics: Investigation of Phenylalanine-Related Metabolic Response in Wheat Upon Treatment With the Fusarium Virulence Factor Deoxynivalenol. Front. Plant Sci. 2019, 10. [CrossRef] [PubMed]
99. Baumeister, T.U.H.; Vallet, M.; Kaftan, F.; Guillou, L.; Svatoš, A.; Pohnert, G. Identification to species level
with altered fatty acid profiles. Plant J. 2013, 76, 138–150. [CrossRef] [PubMed]
98. Sun, M.; Yang, Z.; Wawrik, B. Metabolomic Fingerprints of Individual Algal Cells Using the Single-Probe
Mass Spectrometry Technique. Front. Plant Sci. 2018, 9. [CrossRef] [PubMed]
97. Jaschinski, T.; Helfrich, E.J.N.; Bock, C.; Wolfram, S.; Svatoš, A.; Hertweck, C.; Pohnert, G. Matrix-free
desorption ionization mass spectrometry. Metabolomics 2020, 16, 28. [CrossRef] [PubMed]
96. Baumeister, T.U.H.; Vallet, M.; Kaftan, F.; Svatoš, A.; Pohnert, G. Live Single-Cell Metabolomics
95. Borghi, M.; Fernie, A.R. Outstanding questions in flower metabolism.
94. Selinski, J.; Scheibe, R. Pollen tube growth: Where does the energy come from?
93. Wan, X.; Wu, S.; Li, Z.; An, X.; Tian, Y. Lipid Metabolism: Critical Roles in Male Fertility and Other Aspects
of Reproductive Development in Plants. Mol. Plant 2020, 13, 955–983. [CrossRef]
92. Ken ¯del, A.; Zimmermann, B. Chemical Analysis of Pollen by FT-Raman and FTIR Spectroscopies.
91. Shewry, P.R.; Wan, Y.; Hawkesford, M.J.; Tosi, P. Spatial distribution of functional components in the starchy
dosperm of wheat grains. J. Cereal Sci. 2020, 91, 102869. [CrossRef] [PubMed]
90. Diehn, S.; Zimmermann, B.; Tafintseva, V.; Seifert, S.; Ba˘ gcıo˘ glu, M.; Ohlson, M.; Weidner, S.; Fjellheim, S.;
Mikhael, A.; Jurcic, K.; Schneider, C.; Karr, D.; Fisher, G.L.; Fridgen, T.D.; Diego-Taboada, A.; Georghiou, P.E.;
89. Li, F.-S.; Phyo, P.; Jacobowitz, J.; Hong, M.; Weng, J.-K. The molecular structure of plant sporopollenin.
88. Shewry, P.R.; Wan, Y.; Hawkesford, M.J.; Tosi, P. Spatial distribution of functional components in the starchy
dosperm of wheat grains. J. Cereal Sci. 2020, 91, 102869. [CrossRef] [PubMed]
87. Feenstra, A.D.; Alexander, L.E.; Song, Z.; Korte, A.R.; Yandeau-Nelson, M.D.; Nikolau, B.J.; Lee, Y.J. Spatial
Mapping and Profiling of Metabolite Distributions during Germination. Plant Physiol. 2017, 174, 2532–2548.
[CrossRef] [PubMed]
86. Shewry, P.R.; Wan, Y.; Hawkesford, M.J.; Tosi, P. Spatial distribution of functional components in the starchy
dosperm of wheat grains. J. Cereal Sci. 2020, 91, 102869. [CrossRef] [PubMed]
85. Rolletschek, H.; Melkus, G.; Grafahrend-Belau, E.; Fuchs, J.; Heinzel, N.; Schreiber, F.; Jakob, P.M.; Borisjuk, L.
Combined Noninvasive Imaging and Modeling Approaches Reveal Metabolic Compartmentation in the
Barley Endosperm. Plant Cell 2011, 23, 3041–3054. [CrossRef]
84. Gorzolka, K.; Kölling, J.; Nattkemper, T.W.; Niehaus, K. Spatio-Temporal Metabolite Profiling of the Barley
Germination Process by MALDI MS Imaging. PLoS ONE 2016, 11, e0150208. [CrossRef]
83. Sturtevant, D.; Dueñas, M.E.; Lee, Y.-J.; Chapman, K.D. Three-dimensional visualization of membrane
phospholipid distributions in Arabidopsis thaliana seeds: A spatial perspective of molecular heterogeneity.
Biochim. Biophys. Acta (BBA) Mol. Cell Biol. Lipids 2017, 1862, 268–281. [CrossRef]
82. Woodfield, H.K.; Sturtevant, D.; Borisjuk, L.; Munz, E.; Guschina, I.A.; Chapman, K.; Harwood, J.L. Spatial
Visualization of Membrane Lipid Distributions in Wheat Endosperm.接口分析 2014, 173, 1508–1521. [CrossRef]
81. Usher, S.; Han, L.; Haslam, R.P.; Michaelson, L.V.; Aziz, M.; Chapman, K.D.; Sayanova, O.; Napier, J.A. Tailoring
seed oil composition in the real world: Optimising omega-3 long chain polyunsaturated fatty acid accumulation in transgenic Camellina sativa. Sci. Rep. 2017, 7, 6570. [CrossRef]
120. Hu, C.; Shi, J.; Quan, S.; Cui, B.; Kleessen, S.; Nikoloski, Z.; Tohge, T.; Alexander, D.; Guo, L.; Lin, H.; et al. Metabolic variation between japonica and indica rice cultivars as revealed by non-targeted metabolomics. *Sci. Rep.* 2014, 4, 5067. [CrossRef] [PubMed]

121. Robaina-Estévez, S.; Daloso, D.M.; Zhang, Y.; Fernie, A.R.; Nikoloski, Z. Resolving the central metabolism of Arabidopsis guard cells. *Sci. Rep.* 2017, 7, 8307. [CrossRef] [PubMed]

122. Töpfer, N.; Braam, T.; Shameer, S.; Ratcliffe, R.G.; Sweetlove, L.J. Alternative CAM Modes Provide Environment-Specific Water-Saving Benefits in a Leaf Metabolic Model. *Plant Cell* 2020. [CrossRef]

123. Kim-Hellmuth, S.; Aguet, F.; Oliva, M.; Muñoz-Aguirre, M.; Kasela, S.; Wucher, V.; Castel, S.E.; Hamel, A.R.; Viñuela, A.; Roberts, A.L.; et al. Cell type–specific genetic regulation of gene expression across human tissues. *Science* 2020, 369, eaaz8528. [CrossRef]

124. Shrestha, B. Ten Major Future Challenges in Single-Cell Metabolomics. In *Single Cell Metabolism: Methods and Protocols*; Shrestha, B., Ed.; Springer: New York, NY, USA, 2020; pp. 219–223.

125. Macaulay, I.C.; Ponting, C.P.; Voet, T. Single-Cell Multiomics: Multiple Measurements from Single Cells. *Trends Genet.* 2017, 33, 155–168. [CrossRef]

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