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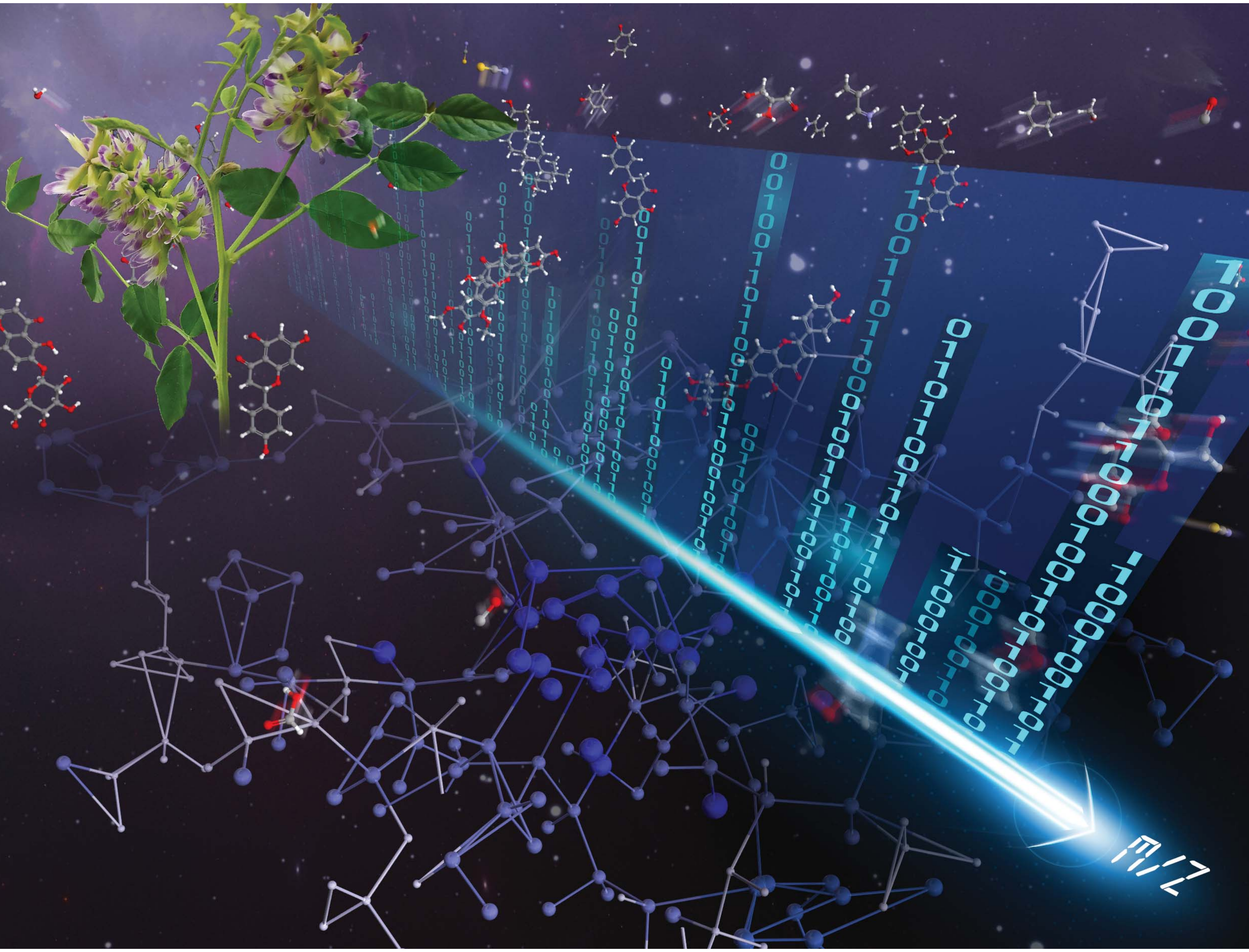

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Metabolomics and complementary techniques to investigate the plant phytochemical cosmos†

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Plants and their associated microbial communities are known to produce millions of metabolites, a majority of which are still not characterized and are speculated to possess novel bioactive properties. In addition to their role in plant physiology, these metabolites are also relevant as existing and next-generation medicine candidates. Elucidation of the plant metabolite diversity is thus valuable for the successful exploitation of natural resources for humankind. Herein, we present a comprehensive review on recent metabolomics approaches to illuminate molecular networks in plants, including chemical isolation and enzymatic production as well as the modern metabolomics approaches such as stable isotope labeling, ultrahigh-resolution mass spectrometry, metabolome imaging (spatial metabolomics), single-cell analysis, cheminformatics, and computational mass spectrometry. Mass spectrometry-based strategies to characterize plant metabolomes through metabolite identification and annotation are described in detail. We also highlight the use of phytochemical genomics to mine genes associated with specialized metabolites' biosynthesis. Understanding the metabolic diversity through biotechnological advances is fundamental to elucidate the functions of the plant-derived specialized metabolome.

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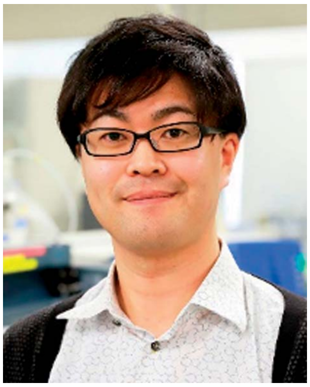
1. Introduction

Plants are predominantly photosynthetic eukaryotes with over 391 000 known species across the planet.[1] One of the fascinating characteristics of plants is their unique metabolic system (metabolism), which produces highly complex and bioactive molecules that modulate the cellular activity, microbiome, and phenotype in human health and diseases.[2,3] Each plant species produces secondary (specialized) metabolites. According to Afendi *et al.*, the plant metabolite diversity exceeds 1 million, with each plant producing nearly 4.7 structurally unique molecules;[4] of these, only ~300 000 structures have been cataloged in the Dictionary of Natural Products (DNP). Moreover,



Hiroshi Tsugawa got his PhD from Osaka University in 2012, Japan. His major is systems biology through computational mass spectrometry (CompMS) to deepen the understanding of metabolisms in living organisms. Employing CompMS, he could develop a data processing pipeline for complex MS data and identify unknown metabolites using computational analysis of mass fragmentation. The awards he received are RIKEN BAIHO Award (2020), Top 40 Under 40 of "The Analytical Science" (2018), and RIKEN Incentive Research Award (2016). Since 2021, he is working as an associate professor at the Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology.

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Amit Rai got his PhD in 2013 from the National University of Singapore. He is currently working as a Research Scientist at the Metabolomics research group, CSRS, RIKEN, and as a guest lecturer at Chiba University. His research interest includes functional genomics for plant natural products using comparative genomics, metabolomics, integrative multi-omics, and systems biology approach. He has been awarded the Young Scientist award at the National University of Singapore (Nov. 2014), best research poster at "The 4th International Conference on Plant Metabolism", Dalian, China (2017), and the best original research by The Japanese Society of Pharmacognosy (2018).

Ryo Nakabayashi received his PhD from Chiba University in 2009, Japan. His expertise is natural product chemistry, analytical chemistry, and metabolomics. As a research scientist, he has joined the Metabolomics Research Group (Principal Investigator: Dr Kazuki Saito), RIKEN CSRS, and has developed metabolomics approaches using LC-MS/MS, MALDI-MS, FTICR-MS, and IMS for identifying specialized metabolites and their functions in plants. He has received the RIKEN Incentive Award in 2017 and the JSPCMB Award for Young Scientists in 2020.

this diversity may be further expanded when human microbial metabolism is considered, where the structure of natural products is modified in the microbiome.[5] In fact, the modified molecule often has higher bioactivity than that of the original form. For instance, 4′,7-dihydroxyisoflavan (equol), catalyzed in the microbiome from the plant metabolite 4′,7-dihydroxyisoflavone (daidzein), acts as a superior ligand for estrogen receptors.[6]

As many metabolites in plants (phytochemicals) possess biologically active sites for mammalian proteins, nearly half of the commercially available drugs released during 1940–2014 were of plant origin.[7] According to the MarketsandMarkets analysis,[8] plant extracts as a commercial commodity in industries, including foods, flavors, cosmetic products, and drugs, were estimated to be worth over USD 23.7 billion in 2019 and have been projected to reach USD 59.4 billion by 2025. Plant specialized metabolites play vital roles in disease resistance, interspecific competition, and stress response (*e.g.*, drought, high light, and abnormal metabolism under nutrient deficiency).[9] More importantly, these metabolites serve pivotal physiological and biological functions to maintain plant homeostasis, suggesting the widely-held view on the difference between primary and specialized metabolites to be obsolete.[10] Therefore, illuminating the structural diversity of plant metabolites is significant for industrial and scientific progress.

Metabolomics, or metabolome analysis, has become popular to explore the biosynthetic pathways of interest and high-throughput screening of natural products.[11,12] Moreover, metabolomics is an essential tool to elucidate the synergistic metabolic pathways of plants and their microbiomes, such as the symbiosis between *Fabaceae* plants and *Rhizobium*.[13] Although next-generation sequencing (NGS) is efficient for obtaining metagenomic information (*i.e.*, the presence of bacterial genes), such static genome information is not always linked to plant phenotypes. In this context, metabolomics provides dynamic information of functional molecules synthesized through the harmonized multi-biomolecule system of metabolites, proteins, RNAs, and epigenomic modifications. Systems biology using multi-omics or transomics data is an active research field to further our understanding of metabolomes.[14,15] Therefore, metabolites and their chemical diversity must be comprehensively studied to elucidate the physiological roles of these metabolites and their underlying molecular mechanisms.

Mass spectrometry (MS)-based untargeted metabolomics has the potential to explore the diversity of plant natural products to study plant metabolism and to perform the high-throughput screening of metabolites. Currently, liquid chromatography coupled with high-resolution tandem MS (LC-MS/MS) is a popular technique owing to the (1) scalability of electrospray ionization (ESI), covering a wide array of chemical properties for metabolite ionization;[16] (2) high mass accuracy (<100 ppb, 1.79 mDa at *m*/*z* 757.52 in 21T FT-ICR) for reliably predicting the molecular formulae of unknown natural products based on the accurate *m*/*z* values of the precursors;[17] (3) information-rich mass fragmentation (recorded as MS/MS spectrum) that provides information on the substructures of the metabolites;[18] and (4) availability of comprehensive metabolite MS/MS spectral libraries (>850 000 unique molecular standards).[19] Moreover, the methodologies of obtaining the retention index using a series of fatty acid-derived chemicals, as used in gas chromatography (GC)-MS, facilitate systematic annotation in reverse-phase and hydrophilic interaction chromatography.[20,21] Furthermore, recent advances in ion mobility (IM) and related informatics tools provide robust and reliable annotation criteria based on the collision cross-section (CCS) values of ∼12 million compounds,[22] thus increasing the coverage of metabolic profiling by separating isobars in a drift tube.[23,24] However, despite the remarkable advances in MS techniques, databases, and informatics tools, the current metabolomics infrastructure in natural product research is inadequate to unveil the global plant metabolome because of the complexity and diversity of the chemical structures, in addition to the lack of MS/MS spectra for most specialized metabolites, particularly alkaloids.

In this review, we focused on the current strategies to explore highly complex and diverse plant metabolomes. First, we provided an overview of the grand challenges of metabolomics in natural product research. The importance of bottom-up approaches such as isolation and/or enzymatic production of plant metabolites is also discussed to clarify the importance of the MS-based metabolomics approach. Second, we reviewed the current MS-based state-of-the-art top-down approaches. Notably, the computational MS (CompMS) techniques aimed at metabolite annotation from the analysis of raw MS datasets were detailed. Third, functional genomics is considered as a complementary module for metabolome analysis, assisting in the discovery of new metabolites and the elucidation of novel metabolic pathways. Finally, we discussed the prospects of plant metabolomics in the coming decade, emphasizing upon a way forward to meet the limitations in the current approaches. Overall, we highlight the importance of integrating MS informatics (CompMS), bioinformatics, and cheminformatics to accelerate plant natural product research.[25]

2. Grand challenges in metabolomics: general approaches to natural product chemistry

Many reviews have focused on the challenges in the comprehensive annotation of natural products using advanced analytical and computational techniques (the top-down approach).[26–28] Most of these techniques aim to increase the rate of “putatively annotated metabolites”; in other words, they identify metabolites based on indirect evidence but do not validate the results using standard compounds. Meanwhile, identifying novel natural products with explicit validation can unveil biological mechanisms hitherto unknown. This can provide novel opportunities for the contribution of plant natural products biochemistry in the field of human medicine. Moreover, such discoveries have been well supported by general biochemical approaches (bottom-up approach), including the isolation of metabolites and production of target compounds using recombinant enzymes or *in vivo* gene transfection.

Herein, we review both the top-down and the bottom-up approaches in metabolomics and the integration of these approaches.

2.1. Annotation in untargeted metabolomics

One of the major challenges in metabolomics is the annotation of diverse molecules.[26] Because small molecules (<2000 Da) show a massive physicochemical diversity, metabolites should be identified based on the match of multiple MS properties, including the retention time, CCS, and MS/MS spectra with those of authentic standards.[18] As a guideline for annotation, Metabolomics Standards Initiative (MSI) has recommended four confidence levels[29] (Fig. 1, Table 1): level 1, identified metabolites using authentic standard compounds; level 2, putatively annotated metabolites using public/commercial spectral libraries; level 3, putatively characterized metabolites based on diagnostic ion and/or partial spectral similarities to known compounds of a chemical class; and level 4, unknown metabolites, although they can still be differentiated and quantified based on the MS profiles. In 2014, Schymanski *et al.* proposed more reasonable criteria for using high-resolution LC-MS/MS-based metabolomics and exposomics as follows: level 1, same as the MSI level 1 definition; level 2a, putatively annotated metabolites matching literature or library spectra, with unambiguous spectrum structure match; level 2b, putatively annotated metabolites matching diagnostic MS2 fragments and/or ionization behavior, when no other structure fits the experimental information; level 3, tentative candidate metabolites with evidence for possible structures but insufficient information on the exact structure; level 4, metabolites for which an unequivocal molecular formula can be unambiguously assigned using the spectral information; and level 5, metabolites whose exact mass (m/z) can be measured in a biological sample and that are of specific interest for investigation but lack information to assign even a formula.[30] Notably, the MSI guidelines are expected to be further revised in the near future to make suitable guidelines in metabolite annotation considered for the latest MS advances including ion mobility.[31] While the importance of annotation strategies tackling levels 2, 3, and 4 of Schymanski *et al.*'s criteria has been reported by several reviews and research articles on metabolomics, level 1 chemical assignment is equally important. Therefore, we firstly summarize the importance of level 1 chemical assignment using a general yet effective approach in natural product chemistry, which facilitates not only the discovery of a novel metabolite structure but also the elucidation of metabolites, which contain the same substructure moieties.

2.2. Motivation to analyze authentic standards in untargeted metabolomics

In both definitions, level 1 annotation requires confirmation against authentic standards; however, preparing all chemicals of interest are impractical because of the lack of commercially available compounds, the difficulty of complete organic synthesis, and the high cost of commercial natural products. However, analyzing authentic standards has important implications for the chemical assignment in metabolomics. Once a metabolite is identified, its MS/MS spectrum is deposited in libraries or databases for annotation in public databases, such as MassBank,[32] GNPS,[33] and MoNA (https://mona.fiehnlab.ucdavis.edu/). Notably, many MS characteristics of metabolites, such as isotopic patterns, m/z values, signal intensities of precursor and product ions, and the fragmentation behavior of the metabolite structure, can be obtained

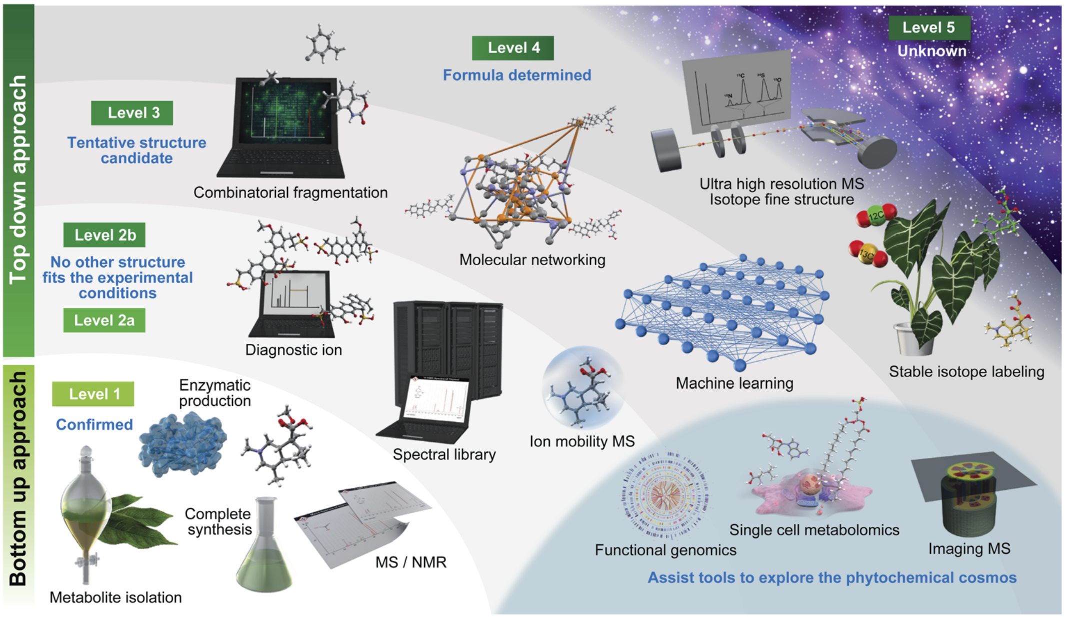

Fig. 1 Roadmap to investigate the diversity of phytochemicals. Essential technologies are described along with the definitions of metabolite annotation recommended by Schymanski *et al.* (2014).[30]

Table 1 Definitions of metabolite annotation proposed by the metabolomics standards initiative and Schymanski *et al.* (2014)[30]

Levels	Metabolomics standards initiative	Levels	Schymanski E. L. *et al.* (2014)
Level 1	Confident 2D structure confirmed by the authentic standard confirming the consistency of retention time and MS/MS spectrum	Level 1	Confirmed structure confirmed *via* appropriate measurement of a reference standard with MS, MS/MS and retention time matching. If possible, an orthogonal method should also be used
Level 2	Putatively annotated structures matched to literature data, mass spectral databases, or other diagnostic evidences in MS/MS spectrum	Level 2a	Library this involves matching literature or library spectrum data where the spectrum-structure match is unambiguous
		Level 2b	Diagnostic represents the case where no other structure fits the experimental information. Evidence can include diagnostic MS/MS fragments and/or ionization behaviour, parent compound information and the experimental context
Level 3	Putatively characterized compound class (unreached to structure level annotation) requiring at least one piece of metabolite information	Level 3	Tentative candidate(s) describes a "grey zone", where evidence exists for possible structure(s), but insufficient information for one exact structure only (*e.g.*, positional isomers)
		Level 4	Unequivocal molecular formula is possible when a formula can be unambiguously assigned using the spectral information
Level 4	Unknown compounds of interest present in biological samples	Level 5	Exact mass (*m*/*z*) can be measured in a sample and be of specific interest for the investigation.

through these resources. Such characteristics are useful to identify the metabolites, even unknown ones, classified in a similar or the same metabolic class based on a unique isotope pattern and/or diagnostic fragment ion matching with a unique substructure. For instance, plants produce multiple isomers of acylsugars, alkaloids, flavonoids (anthocyanins, flavonols, isoflavones, and *C*-flavones), halogen-containing metabolites, iridoids, lignans, phenolamides, phenylpropanoids, saponins, sulfur-containing metabolites (S-metabolites), and terpenoids, and these can be identified using "feature-centric" approaches. The feature-centric approach employs various systematic protocols and software programs to utilize the MS features, including a unique isotopic pattern, fragment ion, neutral loss, and their combination for metabolite annotation: the example is to trace *m*/*z* 285.055 in ESI(+)-MS/MS to grasp metabolites containing kaempferol aglycone.[34–36] Feature-centric characterization is also useful for the dereplication of natural products by removing identified, annotated, and characterized metabolites, resulting in the discovery of truly novel metabolite candidates.[37]

2.3. Compound isolation from plants to identify novel metabolites

To date, orphan receptor elucidation can accelerate medical research and drug discovery once a ligand is identified.[38] Likewise, identifying a novel metabolite structure may lead to the discovery of many metabolites classified in the same metabolite class (or ontology). Therefore, the isolation of truly (dereplicated) unknown compounds from plants of interest is an important step.[39–41]

There are two considerations for unknown metabolite isolation: (1) understanding the physicochemical properties and (2) determining the amount of the starting materials. As described above, MS analysis provides rich information on the physicochemical properties of a metabolite. For instance, when the target ion is highly abundant in either positive or negative ion mode, the chemical property can be considered basic or acidic, respectively. If the ion is equally abundant in both the ion modes, the metabolite is neutral. Notably, at least 100 μg of a compound with high purity must be isolated because structural elucidation often requires nuclear magnetic resonance (NMR) spectroscopy for one- and two-dimensional analyses (up to *m*/*z* 1500). If the target metabolite is likely an isomer of an authentic standard compound, it can be quantified using a standard curve, and the amount of the required plant material can be calculated. However, for truly unknown metabolites, a small-scale experiment must be first performed to predict the concentration of metabolites in the plant and then scaled up as needed. Moreover, the discovery of the plant organ that accumulates a high amount of that metabolite is helpful for effectively isolating the molecule of interest.

Recently, combination approaches using both LC-MS/MS and NMR have been developed to maximize the abilities of metabolite detection of LC-MS and structure elucidation of NMR for MSI level 1 identification.[25,42–44] The use of LC-MS/MS coupled with solid-phase extraction (SPE)-NMR offers an automated system, starting from sample extraction to high-throughput metabolite annotation. Using this approach, over 100 plant-specific metabolites including previously unknown structures have been characterized from *Medicago truncatula*.[44] Importantly, the required compound amount can be reduced to the order of micrograms for the structure elucidation of metabolite analogues.[43] The advantages and limitations of hyphenated MS-NMR systems have been reviewed in detail by several groups.[25,42]

Subsequently, a fractionation strategy was designed based on the knowledge of predicted physicochemical properties of unknown metabolites. In addition to MS, ultraviolet (UV) detection is the most frequently used modality to confirm the fractionation results. Since many metabolites show polarity that corresponds to the carbon backbone structure and functional moieties, the first step is to use liquid–liquid partition to obtain the fraction containing a high amount of the target metabolite; as such, for a highly polar metabolite, a high-polarity solvent (*e.g.*, *n*-butanol and water) is recommended. Moreover, in liquid–liquid partition, solubility in organic solvents with a low polarity (*e.g.*, hexane, ethyl acetate, or chloroform) is employed. Next, the combination of cation and anion exchange chromatography is used to separate the metabolites into three groups: acidic, neutral, and basic. Finally, gel filtration chromatography with resins and a single organic solvent or mixed solvents (*e.g.*, methanol and water) are used to separate metabolites according to their molecular weight. In addition, for further purification, size-based fractions of the metabolites can be separated by reverse-phase chromatography. An efficient way to isolate the target metabolites is to use preparative LC-MS, which can trace the metabolites and isolate it simultaneously by separating a large number of samples injected into the system. Thus, based on the physicochemical characteristics of the target metabolite, a higher rate of isolation can be achieved.

2.4. Enzymatic approaches to obtain natural compounds of interest

The high sensitivity of the MS approach also provides information on the metabolite at trace concentrations in plants. Flavonol glycosides specific to flowers were detected by flavonol profiling using LC-MS but not validated against authentic standards.[45] Flavonols were enzymatically synthesized using *UGT78D3* identified by reverse genetics and transcriptomic analysis. A T-DNA insertion mutant for three possible flavonol glycosides was screened, and two of these, namely, kaempferol and quercetin 3-*O*-arabinoside-7-*O*-rhamnoside, were isolated by chromatography using *in vitro* reaction products with the glycosyltransferase *UGT78D3*. Genome sequencing provides precise information on gene functions but this method is feasible when precursors are commercially available for enzymatic reactions.

2.5. Purposes of metabolite annotation in MSI level 2, 3, and 4 confidences

We summarized the advantages of level 1 identification by a general yet critical plant biochemical approaches in the previous sections. Improving the annotation rates of MSI levels 2 and 3 are an emerging need in metabolomics for the (1) dereplication of natural products, (2) discovery of novel metabolite structures, and (3) elucidation of metabolism in living organisms. The dereplication process can be accelerated by advanced analytical and CompMS[46] techniques that efficiently classify MS ion features to known and expectable (known–unknown) metabolites of MSI levels 1, 2, and 3 and unknown metabolites of MSI level 4. The discovery of new metabolites with the information of a known molecular backbone (aglycone) is also facilitated by untangling the mass spectra, which contain the information of the substructures. In addition, the use of metabolic profiles of level 1 confidence alone is inadequate to understand plant metabolism because <10% of the MS raw data can be annotated by authentic standard-centric annotation.[47,48] Although the isotope- and fragment feature-centric metabolite annotation, which is a knowledge propagation technique,[49] is possible for level 2b and 3 of Schymanski *et al.*'s criteria, such metabolite abbreviated information [*e.g.*, flavonol ($C_{15}H_{10}O_6$) *O*-hex] cannot be utilized directly in either metabolic pathway analysis or integrated genomic, transcriptomic, and proteomic analysis. Therefore, developing bioinformatic tools that can use such structure descriptions, such as plant metabolite-set enrichment analysis,[50] is also an emerging need in plant biology. Such methods have recently been proposed in lipidomics (*e.g.*, lipid ontology enrichment analysis).[51] Recently, a related methodology, where the metabolites are categorized by metabolic pathways and shared tandem MS patterns, has been developed for the interpretation of plant metabolomics data.[52] We believe that the current cheminformatics and CompMS platforms do not meet the requirements of bioinformatics researchers in plant biology compared with the available lipidomics, proteomics, and transcriptomics platforms. Cutting-edge technologies for unknown metabolite annotations have been developed thus far, and the significance of such top-down approaches can be maximized by harmonized integration with general bottom-up approaches.

3. Cutting-edge technologies to identify unknown metabolites

This section focuses on the annotation in metabolomics for natural product chemistry. Here, we describe the current methodologies to enable the annotation of levels 2 and 3 of the MSI guidelines and levels 2a, 2b, 3, and 4 of Schymanski *et al.*'s criteria.

3.1. Technological advances in biology, chemistry, and instrumentation

In this section, technological advances, including stable isotope labeling (SIL), ultrahigh-resolution mass spectrometry (UHRMS), imaging mass spectrometry (IMS), and single-cell metabolomics, are highlighted to show how technology can be used to elucidate unknown metabolites in plants.

3.1.1. SIL-based metabolomics. LC-high resolution (HR)-MS/MS is a popular technique for acquiring metabolomics data. Indeed, the generated accurate masses and informative MS/MS spectra enabled the recognition and characterization of many substructures in unknown metabolites. However, the elemental formula assignment of plant natural products and their substructures remains challenging; in this step, the SIL-metabolomics facilitates the metabolite annotation process by identifying the number of molecular elements (*i.e.*, CHNOS) in unknown precursor and product ions.[53] In the SIL metabolomics approach, the elements of metabolites such as carbon, nitrogen, oxygen, and sulfur are labeled with their stable isotopes (*e.g.*, ^{13}C, ^{15}N, ^{18}O, and ^{34}S). For ^{13}C labeling, $^{13}CO_2$, [U-$^{13}C_6$] glucose, and [U-$^{13}C_{12}$] sucrose are commonly used. ^{15}N or ^{34}S labeling is performed with a liquid fertilizer containing

^{15}N- or ^{34}S-labeled chemicals. The SIL plants produce nearly the same physicochemical properties when compared to the native plants. The labeled and non-labeled compounds are detected based on different *m*/*z* values (observed as mass shifts) according to the number of elements within the same retention time region. Nakabayashi *et al.*[54] showed the landscape of the molecular element changes using principal component analysis (PCA). For the global profiling of plant metabolites in SIL-based metabolomics, the "pair" of labeled and non-labeled metabolites in the LC-MS data can be identified by several CompMS programs, such as MS-DIAL,[55–57] X13CMS,[58] and MetExtract II.[59] The determination of elements helps to predict the molecular formula, substructure, ontology, and even structure accurately, as well as to understand the fragmentation patterns of the complex plant specialized metabolites that are not yet fully deconvoluted.[56] Moreover, SIL-based metabolomics research can be further accelerated using UHRMS data.[60]

3.1.2. UHRMS-based metabolomics. In general, it is difficult to label perennial plants using stable isotopes. For the chemical assignment of unknown plant metabolites, Fourier transform ion cyclotron resonance/magnetic resonance-mass spectrometry (FTICR/MR-MS) is a powerful platform, which provides ultrahigh accuracy and resolution for the metabolite ion (<100 ppb, 1.79 mDa at *m*/*z* 757.52 in 21T FT-ICR) detection.[17] It allows the determination of elements in the precursor and product ions even without using SIL-based metabolomics approaches. In addition, isotopic fine structure analysis,[61] a quantitative and qualitative approach to analyze isotopic ions obtained in UHRMS, can determine the detailed molecular formulas of specialized plant metabolites. The unique *m*/*z* values and signal intensities derived from ^{15}N and ^{34}S isotope elements can be detected in the UHRMS data, and this information is applied to explore nitrogen and sulfur-containing metabolites, which his called N-omics[62] and S-omics,[63] respectively. This methodology can also be applied to oxygen or halogen-containing metabolites. Isotope fine structure analysis is also utilized in MS/MS analysis to detect the substructure ions with the same molecular elements for exploring the target atom-containing metabolites, including the structural isomers.[54]

Nakabayashi *et al.* showed the successful integration of the modern top-down metabolomics approach and the general bottom-up biochemical approach.[64] Asparagus, one of the staple vegetables, is a perennial plant, and it biosynthesizes S-containing metabolites, including asparagusic acid in addition to many unknowns. Sulfur has the stable isotope ^{34}S, which is abundant in nature (4.29%), suggesting that exploration using the differences in the *m*/*z* values between ^{32}S and ^{34}S (1.9958 Da) can easily reveal unknown S-metabolites. In this study, the metabolome data using LC-FTICR-MS were acquired, and ^{34}S-specific isotope features were obtained for all the detected ions. The ion at *m*/*z* 307.08931 ($[M + H]^+$, calculated for $C_{10}H_{19}N_4O_3S_2$, 307.08930) was detected in these data. The search in the databases returned no results for $C_{10}H_{18}N_4O_3S_2$, suggesting that the ion is a new metabolite. Because the metabolite was not detected by UV chromatography, no chromophore in its structure was expected. Moreover, the metabolite was well detected in both positive and negative ionization modes with a high signal intensity, leading to the hypothesis that the metabolite forms a zwitterion. Raw asparagus tissues (970.7 g) were prepared for the isolation of this metabolite as a small-scale experiment. Liquid–liquid partition, excluding low-polarity metabolites and reverse-phase chromatography, successfully obtained a rich fraction including the targeted metabolite. After purifying 61 mg of the compound by preparative LC-MS, a new asparagus metabolite, named asparaptine, was identified by NMR spectroscopy and acid hydrolysis.

3.1.3. IMS metabolomics. IMS is used to characterize the localization of metabolites in cross or longitudinal sections. Generally, both ESI and matrix-assisted laser desorption/ionization (MALDI) can be used in IMS analysis. The advantage of ESI-IMS, as performed in desorption electrospray ionization (DESI),[65] is that the ion features are compatible with LC-ESI-MS/MS, and the annotated metabolites can be directly mapped to the ESI-IMS data, and *vice versa*. MALDI can detect a wide range of metabolites, basically as singly charged molecules, and some ionized molecules are compatible with ESI-MS.[62] A matrix reagent must be sprayed onto the section to extract metabolites from the surface and crystallize both the extracted metabolites and reagents. The reagent assists the ionization of the extracted metabolites in laser irradiation.

Recent IMS analyses have revealed the tissue specificity of plant metabolites.[66] Segmentation analysis,[67] an unsupervised spatial pattern analysis of the detected metabolites, provides a unique localization pattern for a group of metabolites, which are highly accumulated in certain tissues or organs of plants. This indicates that the tissue accumulating the target metabolite of interest can mainly be used for isolation. In fact, the spatial multi-omics approach integrating spatial transcriptomics,[68–70] proteomics,[71] and metabolomics accelerates the understanding of tissue-specific molecular mechanisms, metabolic pathways across tissues, and physiological roles in a plant phenotype.

SIL-assisted spatial metabolomics also facilitates the elucidation of unknown metabolites. Theoretically, both labeled and non-labeled metabolites are localized in the same tissue or organ of the labeled and non-labeled plants, respectively. The *m*/*z* values of the non-labeled metabolites should not be detected in the labeled plant and *vice versa*. The accumulation pattern of the labeled and non-labeled metabolites can be utilized to decrease the false positive annotations and facilitate molecular formula predictions by identifying the number of elements in the IMS data.

3.1.4. Single cell metabolomics. The organ and tissue specificity of the metabolites provide insights into their cell specificity. In the development process of plants, the metabolite levels remain in a dynamic state and play various roles in cells. Single-cell metabolomics is an approach to analyze metabolites at the single-cell level. Live single-cell mass spectrometry (Live-MS) can characterize the cell specificity among the pith, cortical, mesophyll, and epidermal cells in a section.[72] Internal electrode capillary-pressure probe electrospray ionization-MS (IEC-PPESI-MS) revealed the presence of flavonoids and acylsugars in the stalk and glandular cells of the trichomes in *Solanum lycopersicum* (tomato).[73] A combination of IMS and single-cell analysis revealed the movement of alkaloids among the cells in *Catharanthus roseus*.[74,75] Single-cell analysis works well for the outer cells, which are easy to inject with a needle. General metabolomics can be applied as single-cell analysis if the organelles and vacuoles are large. The single-cell approach requires the injection of a needle to draw the cellular components. However, there are concerns regarding the needle destroying the cell walls and membranes of the target and neighboring cells. The contamination of metabolites from the destroyed cells is another concern. Nowadays, cell sorting systems have been developed for single-cell transcriptomics.[76] Metabolites specifically accumulate in certain tissues and cells, suggesting that they play specific roles in these compartments. Combining single-cell technologies can help elucidate the specific roles of metabolites, which remain largely unknown.

3.2. Cheminformatics and CompMS

Computational science is essential to accelerate research in biology. The term "bioinformatics" is popular and widely used when researchers (particularly biologists) state the importance of informatics in biology. However, metabolomics is an interdisciplinary area integrating biology, physics (MS), and chemistry. Therefore, the use of bioinformatics alone is not sufficient to properly represent each computational science in metabolomics. In this context, three terminologies, namely, MS informatics (CompMS), cheminformatics (computational chemistry), and bioinformatics (computational biology) should be used when considering the informatics of metabolomics (Fig. 2). In this section, we focus on the advances in CompMS and cheminformatics.

3.2.1. Generalist, well maintained, and widely used software programs in metabolomics. Many software programs have been developed for metabolomics data processing and analysis.[77] Except for the detailed annotation, analyses such as peak picking, general spectral annotation, alignment (data integration), visualization, and data statistics have currently become simple, thanks to the popular software programs in metabolomics and lipidomics. Although commercial softwares such as MetaboScape, Compound Discoverer, MassHunter, XCMSPlus, and Progenesis QI are superior to the academic ones in terms of stability, well-designed graphical user interface (GUI), and customer support, academic software development is indispensable to rapidly handle the needs of the scientific community, to expand the scientific field to many disciplines, and to reflect the unique ideas of researchers, which can be implemented in commercial softwares in the future. However, even in academia, software programs must be well maintained, tutorialized, and distributed by the developers;[78] otherwise, this development is only helpful for doctoral students and postdoctoral researchers to enhance their academic achievements. Regarding MS coupled with chromatography, MS-DIAL,[55–57,79] XCMS,[80] XCMS-online,[81] MZmine 2,[82] OpenMS,[83] GNPS,[33] and MetaboAnalyst[84] are undoubtedly the most used software programs because of their stability, GUI design, maintenance, and user support (Table 2). These programs have unique features and functions, and third-party programs can complement their shortcomings. MS-FINDER[85] is the buddy program of MS-DIAL to elucidate unknown mass spectra without any reference spectral information while the MS-DIAL program annotates metabolites by existing spectral libraries. MS-CleanR[86] and MS-FLO[87] were designed to curate the metabolome data from MS-DIAL to annotate and group adduct-, isotope-, and in-source fragment ions and to filter the aligned peaks out by several thresholds. The metabolite annotation programs of MetDNA,[88] MetFamily,[89] and GNPS support the output of the MS-DIAL software program. The automated data analysis pipeline (ADAP)[90] for spectral deconvolution is implemented in MZmine 2. GNPS supports the outputs from various software programs such as MS-DIAL, XCMS, MZmine 2, and OpenMS. Furthermore, the GNPS environment contains various attractive programs such as MASST[91] (searching the spectrum across public spectra), ReDU-MS2 (ref. 92) (reanalysis of metabolome data for discoveries), and MS2LDA-MotifDB[93] (finding MS/MS motifs for structure elucidation). As such, the preferred program should be used for the pipeline of metabolomics workflow considering its advantages and disadvantages. However, further development is warranted for the annotation of unknown metabolites.

3.2.2. Mass spectral databases for annotation. The best practice for annotating natural products is to use an MS/MS spectral library. In this review, we investigated the current coverage of MS/MS spectral databases across the chemical cosmos of currently known natural products. As MS/MS databases, we used PlaSMA,[56] MassBank,[32] GNPS,[33] ReSpect,[94] KI-GIAR,[95] CASMI2016,[96] MassBankEU (https://massbank.eu/MassBank/), MetaboBASE,[97] NIST20 (https://www.sisweb.com/software/ms/nist.htm), BMDMS-NP,[98] RIKEN OxPL Library,[99] and FiehnHILIC, PathogenBox, Vaniya/Fiehn Natural Products

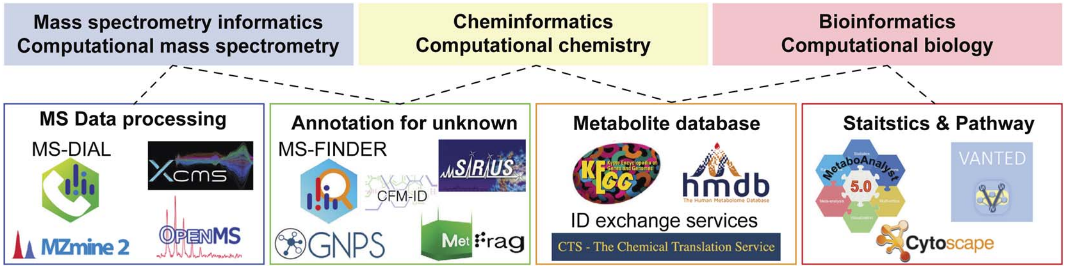

Fig. 2 Links among various informatics areas and the related computational programs in metabolomics. Not all tools are described, and advances are warranted.

Table 2 Summary of the most widely used generalist tools in metabolomics

Software	Platform	Description as of 2020
GNPS	Web	Supporting annotation of unknown EI-MS and MS/MS spectra by many molecular networking techniques. Sharing natural product mass spectra is accelerated. The result of data processing programs from *e.g.* MS-DIAL and MZmine 2 can directly be analyzed in GNPS environment
MetaboAnalyst	Web/local server	Supporting statistics, visualization, and multi-omics analyses for metabolomics data. The metabolome data table for MetaboAnalyst is often prepared by other data processing software tools while it can also be performed in the web application
MS-DIAL	OS free based on C#.net standard environment	The most recently developed of the programs presented. Supporting many data processing pipelines for GC-MS, LC-MS, ion mobility, and data independent acquisition in addition to the intuitive GUI environment and statistics analyses. Functions for curating the results of annotation and alignment are substantial, and many third-party programs including GNPS and MetFamily support the MS-DIAL output
MZmine 2	OS free based on Java Environment	Supporting many data processing functions for GC-MS and LC-MS data in addition to data visualization and basic statistics analyses. The parameter optimization can easily be performed in each of processing module, and the direct links to other software programs like GNPS and Sirius have also been supported by many developers
OpenMS	OS free based on C++ and python environment	Providing an infrastructure of metabolomics and proteomics data analysis workflow by a wide range of customizable tools and functions. With KNIME and Galaxy environments, flexible and scalable workflow can be built. In addition, many data visualization and statistics approaches are supported
XCMS	OS free based on R environment	The first platform of metabolomics data processing. Many informatics researchers contribute to the function developments. Because it is an R package program, the biggest advantage of XCMS is that the result can easily be incorporated to well-maintained bioinformatics tools in the R environment
XCMS-online	Web	Providing an easy-to-use environment for metabolomics data processing. Many default parameter settings are available as a starter for each vendor's machine data. With Metlin, statistics, and pathway platforms maintained in Scripps, it provides the state-of-the-art systems biology platform using metabolomics data

Library of MoNA (https://mona.fiehnlab.ucdavis.edu/): these MS/MS spectra have been experimentally obtained (not as *in silico*). These databases contain 1 304 633 ESI(+)-MS/MS and 367 612 ESI(−)-MS/MS spectra of 36 876 unique structures by the first layer of InChIKey.[100] In the metabolite structure databases, we used information in MS-FINDER,[85] which includes 354 438 unique structures, also curated by the first layer of InChIKey, from HMDB,[101] SMPDB,[102] LipidMAPS,[103] YMDB,[104] ECMDB,[105] BMDB,[106] DrugBank,[107] FooDB (https://foodb.ca/), PlantCyc,[108] ChEBI,[109] T3DB,[110] STOFF-IDENT (https://www.lfu.bayern.de/stoffident/#!home), Blood Exposome DB,[111] Natural Products Atlas,[112] KNApSAcK,[4] NANPDB,[113] UNPD,[114] and biomolecule subspace of PubChem[115] (of note, at the time of writing this review, structures of the COCONUT[116] database were imported to MS-FINDER, and the total number of structures increased to 555 975 in the latest version). The chemical ontology of these structures was defined by the superclass and direct parent terms of ClassyFire.[117] The statistics are shown in Fig. 3a. Importantly, the curated databases for metabolomics do not cover all of the metabolites reported in the literature. Moreover, the metabolite database of MS-FINDER does not cover all the metabolites that have been reported in scientific papers. To perfectly perform statistics of database coverages in the future, the community-based approach,[112] in addition to the database collection approach,[118] are needed, while the important statement of this review is evidenced by the statistics of Fig. 3a.

The results showed the heterogeneity of the chemical cosmos, with only six chemical classes covering 95% of the known metabolites. This may be because (1) the top six groups are the major components of animal and plant cells, (2) the minor chemical classes (<5% of the entire chemical space) are chemically complex and difficult to elucidate, and (3) the definition of chemical classes used is the chemical ontology superclass, which is often broadly defined. For (2), the statistics of the product ion peak abundances were examined (Fig. 3b). Although the inconsistency of the spectral record numbers should be considered, the average product ion peak count per compound was mostly equal among the chemical classes. The only exception is alkaloids (42.6 peaks per compound), lignans (46.4 peaks per compound), organic polymers (96.0 peaks per compound), and hydrocarbon derivatives (61.3 peaks per compound). Our investigation showed that in these four chemical classes, the characteristic ion features to define the aglycone and/or unique substructure moiety were not observed, and the mass fragmentation patterns were difficult to interpret, resulting in the difficulty of comprehensive structure elucidation. Moreover, the statistics of relative intensities, assuming that a high intensity can help us efficiently elucidate and curate structures, also showed no major difference among the chemical classes. This result would indicate that the number of spectral records for minor chemical classes is not sufficient to elucidate the entire chemical space of each chemical class by (1) mass spectrometry specialist-centric manual assignment; (2) characteristic ion-centric knowledge propagation technique, as used in GNPS[33] and MS-DIAL[55] environments; (3) machine learning techniques, as used in SIRIUS,[119] CANOPUS,[120] and CFM-ID;[121,122] and (4) other combinatorial techniques, such as MS-FINDER[85] and MetFrag.[123,124] Importantly, even though authentic standards for a certain chemical class are not available, the spectra would become more informative if the records of available standards are acquired under several conditions, resulting in the better performance of existing tools even for minor chemical classes.

3.2.3. Fragment ion curations to accelerate the substructure annotations. We believe that the most important step, by either manual or computation analysis, is to effectively discover the (sub)structure-specific product ion and/or neutral loss for annotating natural products of level 2b according to Schymanski *et al.*'s criteria.[30] In this review, we assigned substructure ions to each product ion (Fig. 4), wherein multiple records for one compound were merged before assignment according to a previously reported method.[56] In the ESI(+)-MS/MS records of 22 868 compounds, benzenylium (*m*/*z* 77.0386; $C_6H_5^+$) ions were observed in 5648 compounds. Because there are 13 587 compounds containing the benzene moiety in the spectral database, this result indicates that 41.6% benzene-containing molecules generate benzenylium ions in the positive ion mode. As a nitrogen-containing substructure, anilinium (*m*/*z* 93.0573; $C_6H_7N^+$) ion was observed in 1052 compounds and the frequency was calculated to be 20.2% (1052 in 5197 compounds). The most frequently observed neutral loss in the positive ion mode was water loss (18.01 Da; H_2O), indicating the neutral loss of water in 54.2% molecules (7496 in 13 830) containing hydroxyl moiety denoted as "–[OH]" in the simplified molecular-input line-entry system (SMILES) arbitrary target specification (SMART) format. Interestingly, neutral loss of a lysine moiety (146.1055 Da; $C_6H_{14}N_4O_2$) was observed in 83% compounds with a lysine moiety. Moreover, phenolate (*m*/*z* 93.034; $C_6H_5O^-$) and phosphate (*m*/*z* 78.959; PO_3^-) ions were observed in the substructures of 74.4% and 47.4% molecules, respectively.

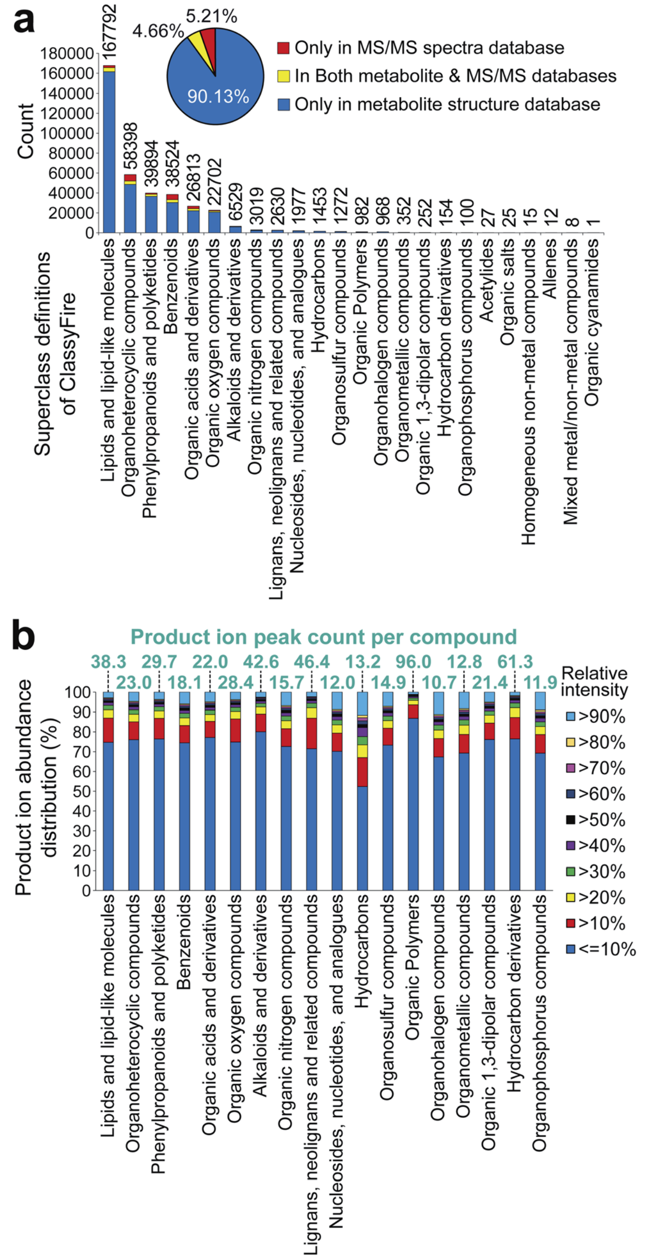

Fig. 3 Statistics of metabolite structure and tandem mass spectral database. (a) The record statistics of the metabolite structure and mass spectra were examined. The metabolite structures implemented in MS-FINDER were used for the statistics, and the detail of spectral records was described in the main text. The chemical ontology was defined by the superclass term of ClassyFire. The number above each bar chart shows the count of unique structures defined by the first layer of InChIkey. The red, yellow, and blue colors indicate structures only contained in the MS/MS databases, structures contained in both spectral and metabolite databases, and structures only contained in the metabolite structure databases, respectively. (b) Statistics of the MS/MS peak count and the relative abundance per compound were examined. Statistics was performed using all the spectral records described in (a). The chemical ontology was defined by the superclass term of ClassyFire.

These statistics are particularly useful to elucidate unknown mass spectraI addition, the specificity of the fragment ion-based substructure elucidation can be increased by the co-existence of related fragment ions, suggesting the existence of a targeted substructure; for instance, the observation of both *m*/*z* 96.969 (HPO_4^-) and *m*/*z* 78.959 (PO_3^-) strongly suggests the existence of a phosphate substructure, although the product ion of *m*/*z* 96.96 is also detected in compounds containing a sulfate moiety (*m*/*z* 96.960; HSO_4^-). The importance of the co-existence

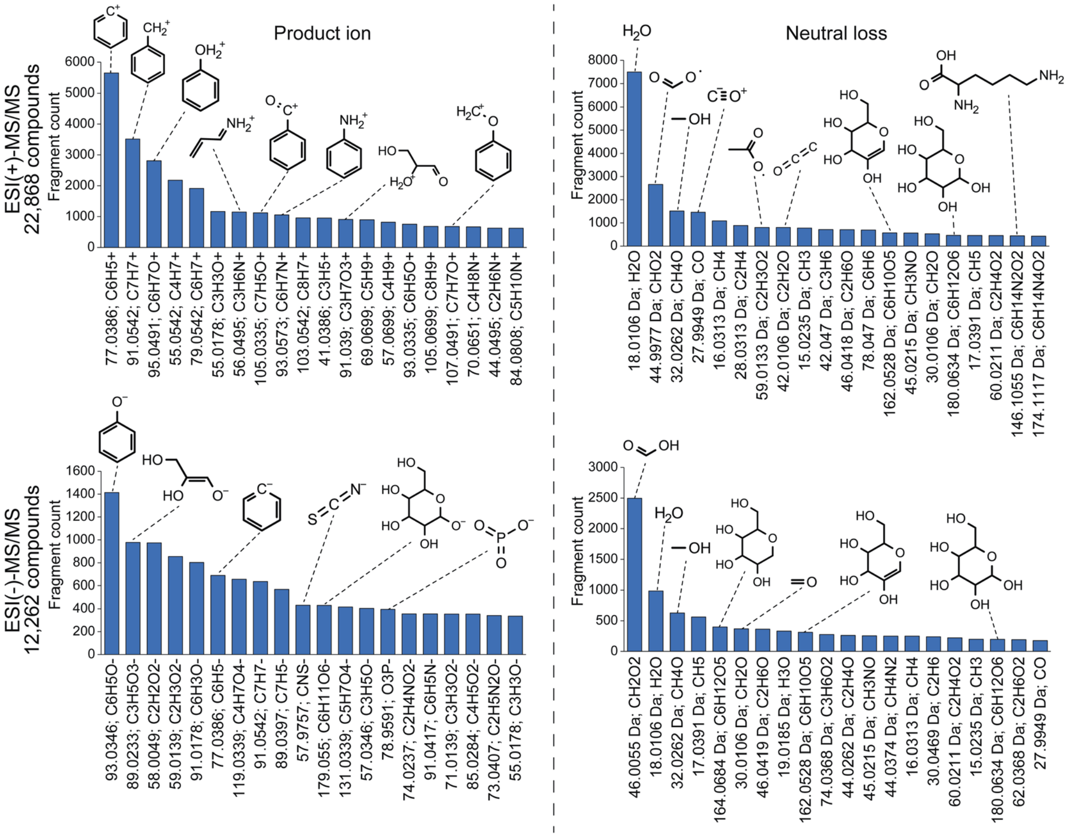

Fig. 4 Statistics of fragment ions and neutral losses. The molecular formula and substructure were assigned using MS-FINDER. Before assignment, multiple records for the same metabolite were merged as a single query.

of fragment ions during metabolite annotation has also been demonstrated and discussed previously by the implementation of topic modelling for metabolomics data with MS2LDA.[36] Furthermore, the relevance of the substructure (even the entire structure) and fragment ions (both product ion and neutral loss) can be investigated by machine learning techniques, such as deep learning and support vector machine (SVM), to increase the specificity, as used in SIRIUS 4[119] and CFM-ID.[121,122] Machine learning to elucidate unknown spectra is currently an active research field as the training set of the substructure-fragment pairs can be easily obtained using combinatorial tools such as MetFrag,[123,124] MAGMa,[125] and MS-FINDER.[85] Meanwhile, the accuracy of structure elucidation tools may be saturated in the near future unless the spectral databases are expanded. Moreover, we emphasize the importance of improving the accuracy of fragment annotation tools rather than structure elucidation tools to increase the quality of the training dataset; in other words, the reliability of the substructure-fragment ion pairs should be improved to increase the accuracy of structure elucidation tools.

3.2.4. Fragment feature-centric metabolite annotations.

The well-elucidated relationship between the MS fragment and the substructure of the metabolites is fundamental to the characterization of plant metabolites within a chemical class (Fig. 5). For instance, flavonoid *O*-glycosides, such as flavanone, flavonol, and (iso)flavone, generate a unique aglycone fragment ion in conventional collision-induced dissociation (CID)-based mass fragmentation.[56,126] Since the relative intensity of such fragment ions often exceeds 80% of the base peak, the aglycone ions of flavonoid *O*-glycosides can be used to elucidate the chemical diversity of glycosides, including glycosyl, galactosyl, rhamnosyl, acetylglycosyl, and malonylglycosyl sugar moieties. In a recent study, we summarized such characteristic product ions and/or neutral losses and used these features to elucidate unknown MS/MS spectra in plant metabolomics data.[56] In fact, the MS fragment-centric chemical assignment enables reliable annotation when the (1) peak intensity of the aglycone-related fragment ions is high in the MS/MS spectrum, (2) mass fragmentation patterns can be easily and intuitively interpreted, and (3) fragmentation patterns are similar within a chemical category; we found that flavonoid *C*-glycosides, prenylated flavonoids, benzoxazinoids, triterpene saponins, glycoalkaloids, steroidal saponins, phenylpropanoids, and iridoids meet these criteria (Fig. 5).

3.2.5. Integrated CompMS and cheminformatics approach to metabolomics.

When the MS/MS spectra of metabolites cannot be elucidated by manual investigation or other combinatorial tools, molecular spectrum networking is useful to obtain unbiased information of tandem mass spectra, assuming that similar structures generate similar MS/MS spectra or share the same fragment ions or neutral losses.

In this review, we performed molecular networking for alkaloids, a chemical class that is difficult to elucidate because of the highly complex mass fragmentation behaviors (Fig. 6): the source data is available as ESI Data.† We used the tandem mass spectra of the PlaSMA database to derive the molecular networks, the methodology of which together with the database details have been described in the previous report.[56] A total of 135 alkaloids were mapped in a network, where metabolites were linked by the similarity of structures (red color edge) and the MS/MS spectra (blue color edge). All the MS/MS spectra analyzed for Fig. 6 were acquired under the same analytical condition. The MS/MS similarity was calculated in the MS-DIAL program using the modified Bonanza score.[127] The source code and the MS/MS database are freely available at the RIKEN PRIMe website (http://prime.psc.riken.jp/). The structure similarity (blue edge) was calculated by the Tanimoto (Jaccard) index to the fingerprints of MACCS,[128] PubChem (https://ftp.ncbi.nlm.nih.gov/pubchem/specifications/pubchem_fingerprints.txt), CDK,[129] and Klekota-Roth.[130] Interestingly, many unique MS/MS patterns were identified in each alkaloid subclass. For instance, tropane alkaloids, ornithine metabolites biosynthesized in *Solanaceae* and *Erythroxylaceae*, generate a product ion at *m*/*z* 124.1121 ($C_8H_{14}N^+$), which matches the tropane backbone. Indolizidine alkaloids generated the product ion at *m*/*z* 160.0757 ($C_{10}H_{10}NO^+$), and yohimbine and corynanthean alkaloids generated product ions at *m*/*z* 174.0913 ($C_{11}H_{12}NO^+$). Moreover, many monoterpene indole alkaloids (MIAs) generated product ions at *m*/*z* 144.0808 ($C_{10}H_{10}N^+$) matched to the indole moiety, and the fragment ion was detected in the MS/MS spectra of >50% of MIAs, denoted by circles in Fig. 6. Furthermore, the molecular

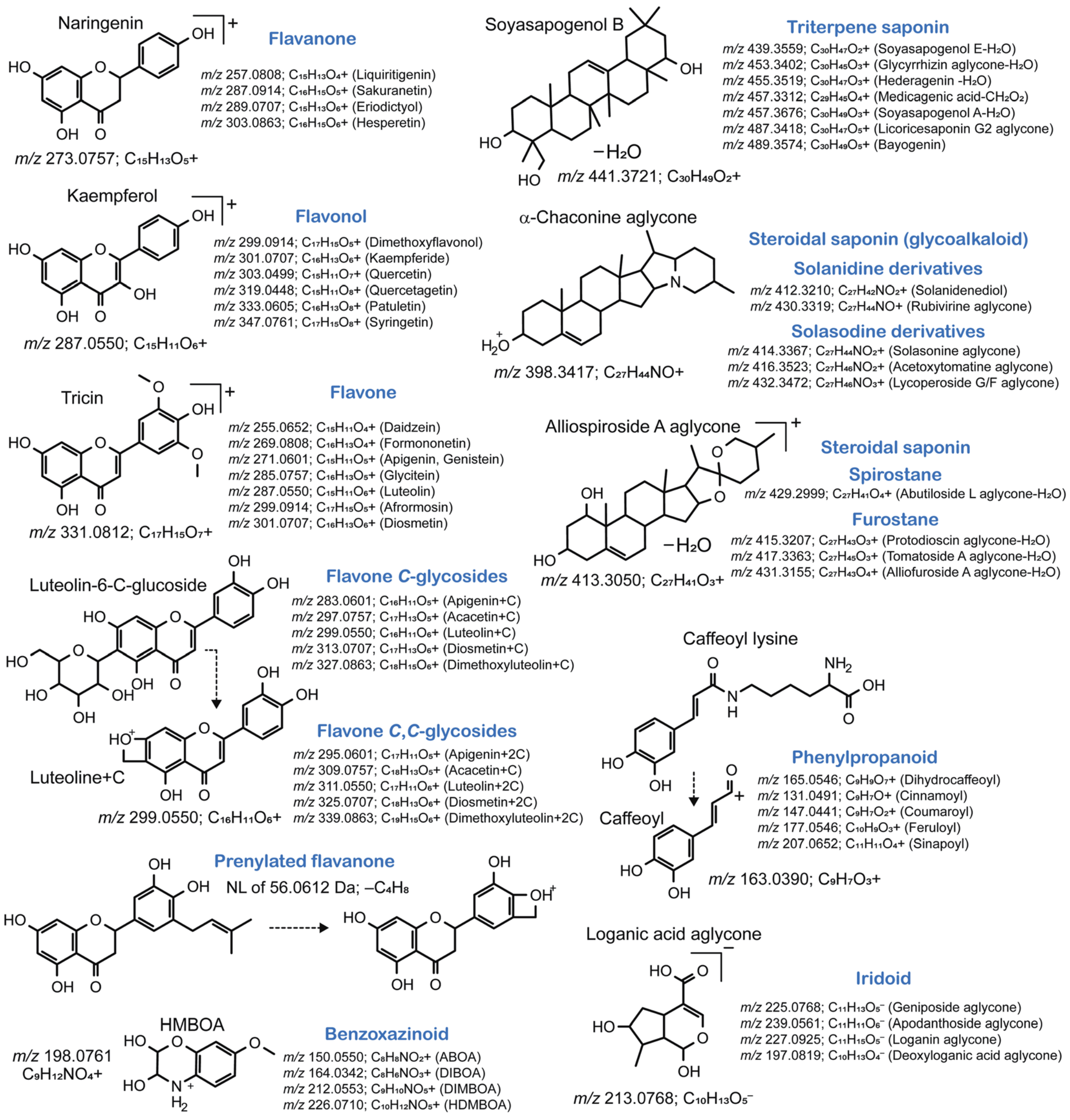
Naringenin
Flavanone
m/z 257.0808; C15H13O4+ (Liquiritigenin)
m/z 287.0914; C16H15O5+ (Sakuranetin)
m/z 289.0707; C15H13O6+ (Eriodictyol)
m/z 303.0863; C16H15O6+ (Hesperetin)
m/z 273.0757; C15H13O5+
Kaempferol
Flavonol
m/z 299.0914; C17H15O5+ (Dimethoxyflavonol)
m/z 301.0707; C16H13O6+ (Kaempferide)
m/z 303.0499; C15H11O7+ (Quercetin)
m/z 319.0448; C15H11O8+ (Quercetagetin)
m/z 333.0605; C16H13O8+ (Patuletin)
m/z 347.0761; C17H15O8+ (Syringetin)
m/z 287.0550; C15H11O6+
Tricin
Flavone
m/z 255.0652; C15H11O4+ (Daidzein)
m/z 269.0808; C16H13O4+ (Formononetin)
m/z 271.0601; C15H11O5+ (Apigenin, Genistein)
m/z 285.0757; C16H13O5+ (Glycitein)
m/z 287.0550; C15H11O6+ (Luteolin)
m/z 299.0914; C17H15O5+ (Afrormosin)
m/z 301.0707; C16H13O6+ (Diosmetin)
m/z 331.0812; C17H15O7+
Luteolin-6-C-glucoside
Flavone C-glycosides
m/z 283.0601; C16H11O5+ (Apigenin+C)
m/z 297.0757; C17H13O5+ (Acacetin+C)
m/z 299.0550; C16H11O6+ (Luteolin+C)
m/z 313.0707; C17H13O6+ (Diosmetin+C)
m/z 327.0863; C18H15O6+ (Dimethoxyluteolin+C)
Luteoline+C
Flavone C,C-glycosides
m/z 295.0601; C17H11O5+ (Apigenin+2C)
m/z 309.0757; C18H13O5+ (Acacetin+C)
m/z 311.0550; C17H11O6+ (Luteolin+2C)
m/z 325.0707; C18H13O6+ (Diosmetin+2C)
m/z 339.0863; C19H15O6+ (Dimethoxyluteolin+2C)
m/z 299.0550; C16H11O6+
Prenylated flavanone
NL of 56.0612 Da; –C4H8
HMBOA
Benzoxazinoid
m/z 198.0761 C9H12NO4+
m/z 150.0550; C8H8NO2+ (ABOA)
m/z 164.0342; C8H6NO3+ (DIBOA)
m/z 212.0553; C9H10NO5+ (DIMBOA)
m/z 226.0710; C10H12NO5+ (HDMBOA)
Soyasapogenol B
Triterpene saponin
m/z 439.3559; C30H47O2+ (Soyasapogenol E-H2O)
m/z 453.3402; C30H45O3+ (Glycyrrhizin aglycone-H2O)
m/z 455.3519; C30H47O3+ (Hederagenin -H2O)
m/z 457.3312; C29H45O4+ (Medicagenic acid-CH2O2)
m/z 457.3676; C30H49O3+ (Soyasapogenol A-H2O)
m/z 487.3418; C30H47O5+ (Licoricesaponin G2 aglycone)
m/z 489.3574; C30H49O5+ (Bayogenin)
−H2O
m/z 441.3721; C30H49O2+
α-Chaconine aglycone
Steroidal saponin (glycoalkaloid)
Solanidine derivatives
m/z 412.3210; C27H42NO2+ (Solanidenediol)
m/z 430.3319; C27H44NO+ (Rubivirine aglycone)
Solasodine derivatives
m/z 414.3367; C27H44NO2+ (Solasonine aglycone)
m/z 416.3523; C27H46NO2+ (Acetoxytomatine aglycone)
m/z 432.3472; C27H46NO3+ (Lycoperoside G/F aglycone)
m/z 398.3417; C27H44NO+
Alliospiroside A aglycone
Steroidal saponin
Spirostane
m/z 429.2999; C27H41O4+ (Abutiloside L aglycone-H2O)
Furostane
m/z 415.3207; C27H43O3+ (Protodioscin aglycone-H2O)
m/z 417.3363; C27H45O3+ (Tomatoside A aglycone-H2O)
m/z 431.3155; C27H43O4+ (Alliofuroside A aglycone-H2O)
−H2O
m/z 413.3050; C27H41O3+
Caffeoyl lysine
Phenylpropanoid
m/z 165.0546; C9H9O7+ (Dihydrocaffeoyl)
m/z 131.0491; C9H7O+ (Cinnamoyl)
m/z 147.0441; C9H7O2+ (Coumaroyl)
m/z 177.0546; C10H9O3+ (Feruloyl)
m/z 207.0652; C11H11O4+ (Sinapoyl)
Caffeoyl
m/z 163.0390; C9H7O3+
Loganic acid aglycone
Iridoid
m/z 225.0768; C11H13O5− (Geniposide aglycone)
m/z 239.0561; C11H11O6− (Apodanthoside aglycone)
m/z 227.0925; C11H15O5− (Loganin aglycone)
m/z 197.0819; C10H13O4− (Deoxyloganic acid aglycone)
m/z 213.0768; C10H13O5−

Fig. 5 Characteristic fragment ions to elucidate the metabolites within a chemical class. This figure was prepared based on ESI Table 5† of the previous study.[56]

network suggested that the ion mobility-centric diagnostic criteria described by the CCS value of the structure would increase the confidence of metabolite annotations. In Fig. 6, the CCS value is reflected by the size of the node. Many metabolites with similar MS/MS spectra have distinct CCS values. The CCS values were obtained from the AllCCS[22] and PNNL CCS databases.[131] For structures lacking CCS information, the value was predicted using AllCCS (http://allccs.zhulab.cn/). However, this network also revealed that many alkaloids do not show similar MS/MS spectra and distinct CCS values, even though they are part of the same biosynthetic pathway (*e.g.*, strictosidine, strictosamide, and camptothecin). Further integrative approaches and database accumulation are warranted to elucidate the yet unknown total alkaloid chemical cosmos.

4. Phytochemical genomics in a supporting role to explore the metabolite cosmos

The advances in the form of next-generation sequencing technologies, emerging approaches for assembly scaffolding, and innovative computational tools to achieve chromosome-scale genome assemblies for even a highly repetitive genome content have begun the genomic era for non-model plant species rich in metabolite diversity. Genomics-metabolomics together complement each other to prioritize genes and metabolites for functional and chemical annotations, respectively. In this section, we briefly describe the impact and potential of genomics to complement the advances in new metabolite discovery.

4.1. Importance of genome mining for plant natural product discovery

The emergence and expansion of plant functional genomics are often attributed to multi-omics analyses, which have enabled the prioritization and functional characterization of thousands of genes to date.[132–134] Genes associated with the biosynthetic pathways of specialized metabolites are often coexpressed and strongly correlated with metabolite accumulation, forming a basis for this analytical approach.[135] Nevertheless, the number of genes identified through this strategy is in hundreds, if not in thousands. Therefore, additional criteria to narrow down the candidate genes for functional characterization are essential to predict possible intermediates of specialized metabolite biosynthetic pathways. Genome mining is vital for expanding the microbial natural product discoveries.[28] In addition to the added advantage of a smaller genome size with hundreds of thousands of published microbial genomes as a resource for comparative genome analyses, gene clusters have been established as the key feature of microbial natural product biosynthesis. Thus, an identified gene cluster not only associates genes with biosynthesis but also provides clues into metabolite intermediates based on the enzymatic properties of the genes in

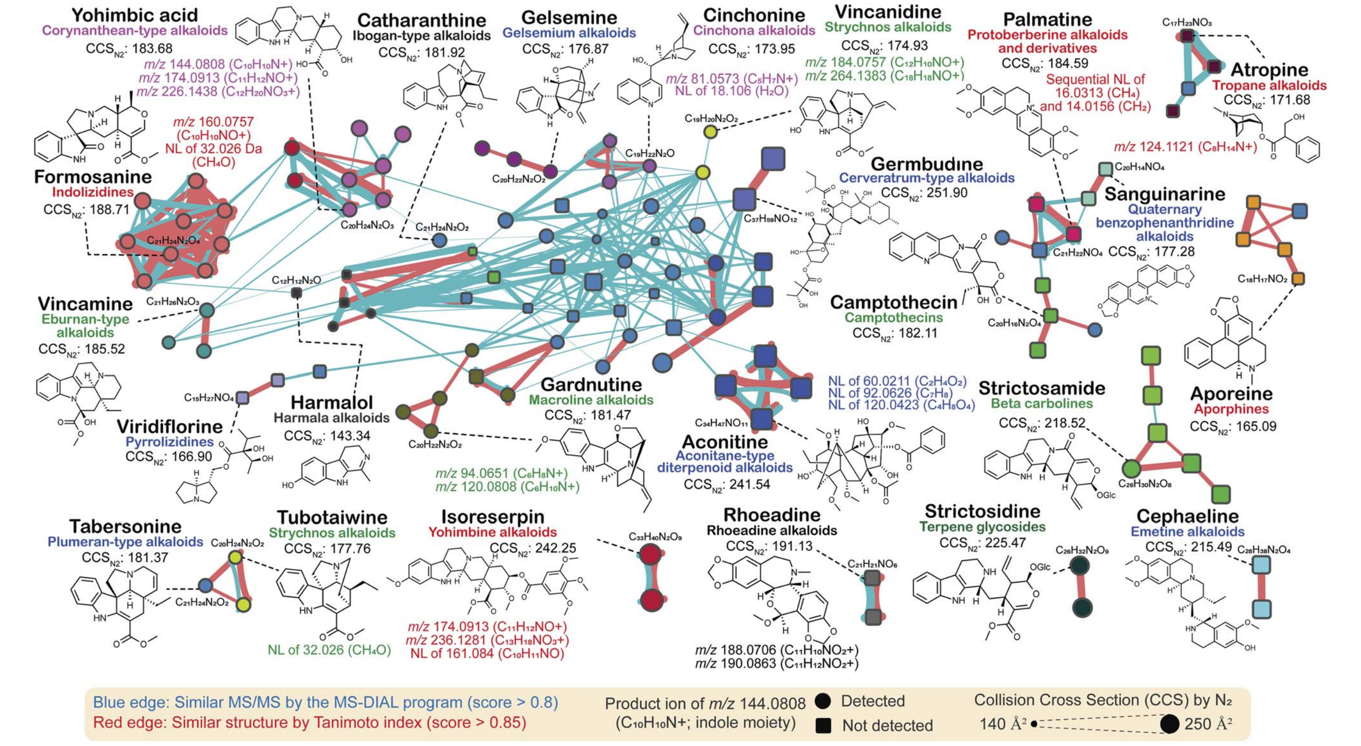

Fig. 6 Molecular networking of 135 alkaloids in the PlaSMA database. The MS/MS spectra were obtained under the same analytical condition. The MS/MS similarity (red edge) was calculated in the MS-DIAL program. The structure similarity (blue edge) was calculated by the Tanimoto index based on the structure fingerprints. The metabolite classification follows the definitions of the direct parent terms in ClassyFire. The same node color means the same direct parent term of ClassyFire.

that cluster. However, compared with the microbial systems, plant genome-based natural product discoveries remain limited because of the near negligible genomic resources available owing to the sheer vastness and diversity of the plant kingdom, their enormous genome sizes, prevalence of repeat-rich genomes, and polyploidy.[132,134]

In recent years, tremendous progress has been achieved in the handling some of the challenges mentioned above owing to a significant reduction in the per-base sequencing cost, advances in long-read sequencing technologies, increased sequencing throughput, and a rapidly expanding toolbox. RNA-Seq-based *de novo* transcriptome assembly has allowed for the generation of genome resources for thousands of plant species in the last few years.[133,134] Consortium-based efforts, such as the 1KP Project, have reported genome resources for 1124 plant species, covering several diverse plant species from distant lineages.[136] The established high-quality genome assemblies further complements these efforts.[135] In addition to individual genome projects targeting specific plants of interest, consortium efforts, such as Earth Biogenome Project[137] and Darwin Tree of Life project (https://www.darwintreeoflife.org/), are aimed at establishing whole-genome assemblies for thousands of diverse plant species in the next decade. These high-quality genome assemblies are particularly valuable for understanding the roles of gene clusters and structural variants, which would be beneficial for modern functional genomics and deep learning tools to predict the functions of unknown components. Here, we briefly elaborate on the current genome-mining approaches and integrative omics approaches to explore natural product biosynthesis (Fig. 7).

4.2. Near-isogenic lines (NILs), recombinant inbred lines (RILs), and chromosome segment substitution lines (CSSLs) for metabolome-assisted functional genomics

NILs, RILs, and CSSLs are valuable genetic resources for identifying genes associated with a given trait. Typically, these lines serve as powerful tools for genetic analysis and characterization of donor varieties or quantitative traits of the species against the genetic background of a recurrent parent[138–146] as well as for the identification of minor-effect quantitative trait loci (QTLs), resulting in the acquisition of novel properties/traits of the donor genotype and identifying the genomic segments and potential genes responsible for a specific trait.[140,141,143,147–149] Moreover, NILs, RILs, and CSSLs are vital genetic resources for identifying the genes associated with novel agronomical properties and specialized metabolism.[144–146,149,150] QTLs associated with metabolites have been identified to detect polygenic regions and genes associated with biosynthetic pathways.[148,150–155] Using 210 RILs, Kang *et al.* identified 4681 putative metabolites associated with QTLs and used *in silico* analysis to characterize 35 candidate genes associated with the biosynthesis of 30 structurally identified metabolites, including genes responsible for the variation in the feruloyl serotonin and L-

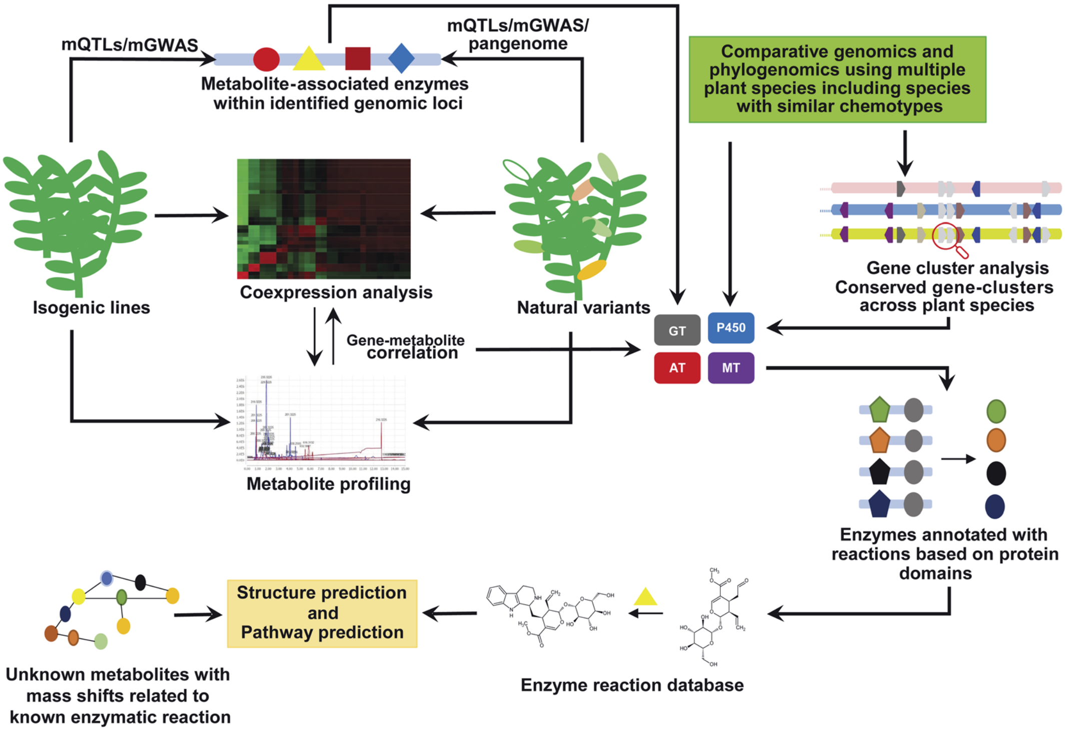
mQTLs/mGWAS
mQTLs/mGWAS/ pangenome
Metabolite-associated enzymes within identified genomic loci
Comparative genomics and phylogenomics using multiple plant species including species with similar chemotypes
Isogenic lines
Coexpression analysis
Natural variants
Gene cluster analysis Conserved gene-clusters across plant species
Gene-metabolite correlation
GT
P450
AT
MT
Metabolite profiling
Enzymes annotated with reactions based on protein domains
Structure prediction and Pathway prediction
Unknown metabolites with mass shifts related to known enzymatic reaction
Enzyme reaction database

Fig. 7 Genome mining to discover and annotate new metabolites. Multi-omics analysis using isogeneic lines and natural variants of a given plant species could be used to identify new metabolites and putatively associated genes involved in its biosynthesis. Using comparative genomics, phytochemical genomics, gene cluster analysis, and using the prior known biochemical reactions will allow to improve the annotation of new metabolites taking into consideration the observed mass-shift and the identified enzyme families across species with similar chemotypes. GT: glycosyltransferases; AT: acyltransferase; MT: methyltransferases; P450: cytochrome P450.

asparagine content across populations.[156] Using a large cross-population of maize and its wild ancestor, teosinte, Xu *et al.* identified genetic factors controlling the metabolic divergence responsible for maize domestication.[157] The authors used integrative omics approaches to identify the candidate genes contributing to metabolite divergence and verified the roles of flavanone 3-hydroxylase1, purple aleurone1, and maize terpene synthase1 in the divergence of their related biosynthetic pathways. Using RILs created by crossing *Arabidopsis* Col-0 and C24, Knoch *et al.* identified 786 metabolic QTLs on the short arm of chromosome 4 responsible for a major proportion of metabolic variation, including potential genes involved in the biosynthetic pathways.[158]

Inbred-line genomics has been particularly successful in the discovery of new metabolites and associated biosynthetic genes in tomatoes. Metabolic QTL analysis across 76 introgression lines of tomato identified 679 genomic regions associated with the specialized metabolism in the fruit pericarp;[150] multi-omics analysis identified the candidate genes associated with the key QTLs. Subsequently, *Solyc06g062290* and *Solyc10g085230*, which are involved in glycoalkaloid biosynthesis, were functionally characterized. Schilmiller *et al.* used CSSLs and a forward genetics approach to identify the diversity of mono- and sesquiterpene biosynthesis and associated QTLs in the secreting glandular trichomes of tomato.[159] The authors identified genomic regions including potential candidate acyltransferases, which regulate the accumulation of total trichome terpenes or acyl sugars, alteration of sesquiterpenes with intact monoterpene moieties, accumulation of the monoterpene α-thujene, and acylsucrose lacking an acetyl group, and shifts in the length of the acyl chains in acyl sucrose. Furthermore, Schilmiller *et al.* functionally characterized the BAHD family of acyltransferases (*Solyc01g105580* or SlAT2), encoding an acetyl-CoA-dependent acyltransferase, and found the addition of acetyl groups to the major detectable tetra-acylsucrose.[160] Moreover, Alseekh *et al.* identified 338 putative metabolite QTLs associated with flavonoids, steroidal glycoalkaloids, and other specialized metabolites using the seeds of *Solanum pennellii* introgression lines. Authors experimentally validated flavonoid-associated QTLs, including *Solyc12g098600* and *Solyc12g096870*, which encode seed-specific uridine 5-diphosphate-glycosyltransferases.[161] In a comprehensive multi-omics analysis using a population of hundreds of diverse tomato accessions, Zhu *et al.* identified thousands of genetic regions associated with metabolism.[162] They showed that the alleles of the genes associated with large fruit were linked to metabolism and identified five major loci that reduced the accumulation of anti-nutritional steroidal glycoalkaloids in ripe fruit. Using an introgression population developed from the wild Peruvian accession of *Solanum pennellii* (LA0716 or PI246502) and the *Solanum lycopersicum* cultivar M82, Szymanski *et al.* established the genetic basis of chemical variations accompanying the transfer of wild-type fruit traits.[163] In this study, integrated genome-transcript-metabolite-phenotype QTL analysis was used to elucidate the biosynthesis of esculeosides and lycoperosides from α-tomatine during fruit development and ripening.

These and several other exceptional studies on *Arabidopsis*, tomato, rice, wheat, soybean, pepper, maize, and potato, among other plant species, have shown a strong association of specialized metabolites with QTLs.[157,158,164–166] These lines also offer a potential resource for identifying the intermediates of a given metabolic pathway. However, the generation of such lines is challenging and requires time and resources. Nevertheless, the advantages of discovering new properties and establishing new metabolites through such lines are promising. Artificially generated genetic variability within a given species through fast-neutron or gamma-ray bombardments and ethyl methanesulfonate (EMS) mutagenesis is an alternative to screen and select lines based on the desired phenotype for further characterization.[167]

4.3. Natural variants for metabolome-assisted functional genomics

Within a given plant species, ecosystem changes result in spontaneous mutations driven by evolutionary processes, such as natural or artificial selection (*i.e.*, domestication), thus deriving natural intraspecific variation (hereafter, natural variation).[133,168] These natural variants are also the main toolsets for plant breeders to establish inbred lines with desired agronomical traits.[169] Natural variants include single-gene (monogenic) allelic variants and, in many cases, even massive changes through transposon-based genome expansions, deletion/expansion of enzymes, and altered regulation of enzymes involved in biosynthetic pathways. The natural variants of *Arabidopsis* have served to identify over 100 genes associated with the adaptation of plants to different natural environments, including transcription factors, hormones, and primary and biosynthetic enzymes.[132,170] In natural variants of *Arabidopsis*, untargeted metabolite profiling identified 18 unknown mass features, including novel flavonol derivative saiginol A, which shows enhanced UV-B absorbent properties compared with other phenylpropanoids.[41] With over 400 000 rice germplasm accessions stored in gene banks worldwide, the metabolite profiling of rice natural variants identified differential levels of aromatic and bioactive metabolites across accessions, contributing to typical characteristics and phenotypes.[152,171,172] Natural variants of crops such as tomato, soybean, maize, potato, peanut, strawberry, and cucumber are being collected and maintained worldwide, offering an excellent resource for identifying new metabolites and their association with the phenotype.[162,169,173,174] The availability of high-quality genome assemblies allows researchers to use natural variants for accumulating molecular evidence, resulting in colossal chemodiversity.

Genome-wide association studies (GWASs) have gained prominence in achieving a high resolution (to the single nucleotide level) and dissect the genetic architecture with the associated traits.[132,174] The advantages of GWAS coupled with metabolomics for large-scale interactive gene-metabolite annotation and identification and metabolic pathway elucidation are well-known.[175,176] The combination of GWAS with untargeted metabolomics of 440 *Arabidopsis* natural variants identified the novel specialized metabolite *N*-malonyl-D-alloisoleucine.[177] GWAS, combined with metabolomics of 529 rice accessions, enabled the identification and functional characterization of 36 candidate genes associated with the specialized metabolism of physiological and nutritional importance.[175] Furthermore, unknown metabolites, including sakuranetin, pyridoxine *O*-glycoside, and phytocassane D, and 166 other metabolites were identified using associations with functionally related genes with this approach. GWAS combined with QTL analysis identified genes involved in specialized metabolism.[178,179] GWAS is increasingly being applied in the combination of QTLs to identify and validate potential single-nucleotide polymorphisms (SNPs) associated with a given trait[180,181] as it allows the precise identification of the genes and genomic regions associated with the trait of interest. Relatively fewer loci often control the metabolic traits with significant effects, making the combination of GWAS with QTL and coexpression analysis a powerful functional genomics approach. Metabolome-associated GWAS analysis can assign unknown metabolites to a specific genomic region, which can be further used in prioritization for further structural validation.[182]

One of the limitations of GWAS, which mainly uses short-read sequencing for mapping to a single reference genome, is the loss of genetic information from highly polymorphic regions due to its dependence on the sequence similarity. A single reference genome for a plant species, given the huge natural variants, also means that it may not include some of the vital genomic segments or genes responsible for specific features.[183,184] Therefore, pangenomes are essential to understand the extent of genomic variation and overall molecular features that characterize a species. A pangenome for a given species mainly includes the core genome, shared across natural variants, and the dispensable genome, genetic part that varies across the participating accessions, including its chemodiversity.[183] The comparison of genomes between the inbred grapevine variety Pinot Noir (PN40024; sequenced in 2007; reference genome) and the grapevine variant Uruguayan Tannat clone (UY11) containing high polyphenol levels in the berry skin and seed showed that 1873 genes were absent in the reference genome.[185] UY11 expressed 141 novel unique genes encoding 19 different enzymes associated with polyphenol biosynthesis, including cultivar-specific genes regulating polyphenol accumulation. The pangenome for tomato, constructed using 725 phylogenetically and geographically distinct accessions, identified 4873 genes absent from the reference genome.[186] Moreover, *TomLoxC* (Solyc01g006540) was revealed to be involved in the production of apocarotenoid, which contributes to the desirable tomato flavor. Pangenome for *Brachypodium dystachyon* showed that the core genome was rich in genes associated with essential processes such as primary metabolite synthesis, while the dispensable genome was rich in genes associated with disease resistance and abiotic stress response.[187] Most importantly, the dispensable genome showed higher synonym substitution compared to the core genome, suggesting ongoing active evolution within these natural variants through these gene sets, including genes associated with metabolism. Pangenomes for *Arabidopsis* and some of the key crops, including maize,[188] soybean,[189] rice,[190,191] medicago,[192] tomato,[186,193] *Brassica napus*,[194] sunflower,[195] wheat,[196] and *Brassica oleracea*,[197] have been constructed in the past few years and are a valuable resource for identifying various genes and their functions, which confer a characteristic phenotype to a given cultivar. The analysis of natural variants can help understand the role of genetic diversity to derive the evolution of specialized metabolites' biosynthesis through means such as SNPs, small insertions/deletions, structure, gene presence/absence, gene copy number, and other miscellaneous genomic features. Pangenome, GWAS, and QTL analysis combined with metabolomics in natural variants provide a means to link unknown metabolites to genes of known function, which can be used to predict and annotate additional metabolites and genes.

4.4. Comparative genomics and phylogenetic approaches to elucidate the metabolites' diversity and role in speciation

Comparative genomics and phylogenomics approaches allow for tracing back and speculate regarding events that drove the evolution of specialized metabolites, including the identification of key genes and metabolites.[179] Evolutionary forces, including (1) localized gene duplication, sub-genome duplication, or whole-genome duplication, followed by sub- or neo-functionalization of specific enzymes; (2) allelic variation; (3) gene loss; and (4) catalytic promiscuity, work cohesively under the influence of positive natural selection to bring large structural diversity in specialized metabolism.[168,198] A generalized scenario for the evolution of specialized metabolites involves the emergence of new enzymes through local, sub-genome, or whole-genome duplications, thus providing gene pools to evolve new functions responsible for chemodiversity.[199,200] In the dynamic evolutionary process, enzyme catalytic promiscuity allows the divergence of the metabolic stream toward enhanced chemodiversity. Gene duplication with enzyme promiscuity, followed by changes in the substrate specificity, has been identified as the mechanism underlying the evolution of the glucosinolate biosynthetic pathway.[201] The catalytic promiscuity of the enzymes, such as acyltransferases, a diverse enzyme family catalyzing *O*-acylation and *N*-acylation of structurally diverse acceptor substrates including alkaloids, phenylpropanoids, terpenoids, and acylsugars, is one of the key driving forces of metabolite diversity.[202] By expressing a bifunctional lysine/ornithine decarboxylase enzyme, L/ODC, in *Arabidopsis*, Shimizu *et al.* showed the emergence of non-native specialized metabolites, including alkaloid-like metabolites.[203] The authors used the core chemical structure of cadaverine to identify the metabolic intermediates and enzymes involved in artificially established chemodiversity in *Arabidopsis*; they demonstrated the role of promiscuous enzymes in deriving the metabolite diversity and described the emergence of metabolite scaffolds as the key event.

Analyzing the genomes of multiple plant species has provided evidence of a rather surprising prevalent convergent evolution for different metabolite classes across the plant kingdom.[204] Comparative genomics using genomes of *Nicotiana attenuata* and *Nicotiana obtusifolia* showed the association of genome evolution with the establishment of nicotine biosynthetic pathways.[205] Caffeine and other purine alkaloid biosynthetic pathways in plants have evolved from several unrelated gene families.[206] The biosynthesis of MIAs, one of the most diverse and economically valuable metabolite classes, has evolved convergently.[207] Using comparative genomics and phylogenomics, Rai *et al.* showed the importance of strictosidine biogenesis in the evolution of camptothecin and other MIA biosynthetic pathways.[207] They compared the genomes of MIA-producing plants and showed that enzymes associated with MIA biosynthesis emerged after the evolution of functional strictosidine synthase (STR). STR loss was associated with the loss of the ability to evolve the cellular components essential for MIA biosynthesis. They also identified the parallel evolution of CPT biosynthesis in distant plant species. Convergent evolution through substrate promiscuity has also been reported for Lys-derived alkaloids, sesterterpenoids, glucosinolates, benzylisoquinoline alkaloids, and tropane alkaloids.[204]

A general analytical pipeline to discover candidate genes involved in specialized metabolism begins with synteny analysis (intra- and inter-species) to identify gene sets that are conserved across the plant species, produce similar classes of metabolites, and have undergone duplication. Such gene sets can be further analyzed for gene family classification, followed by synonymous substitution analysis to identify genes with recent modifications or specialization. Phylogenetic analysis and hypothesis testing using various (HyPhy) tools[208] with prior knowledge of the type of specialized metabolite produced in the target plant species allow the identification of positively selected candidate genes. Comparative genomics and phylogenomics with homology-based annotation predict the potential enzymatic activity and spatial expression patterns in tissues accumulating specialized metabolites as the criteria can further assist in narrowing down the candidate genes for functional characterization.[207] Phylogeny-based enzyme classification allows predicting potential functions of candidate genes, and in extension, metabolite intermediates of the associated enzymatic reactions. Phylogenomics-based plant metabolite structure prediction is not new but the analytical scale is limited to a few candidate structures and requires time and resources. In a recent study, Defossez *et al.* constructed a framework to predict the landscape-scale phytochemical diversity of known and unclassified molecules using an untargeted metabolomics approach on 416 grassland vascular plant species with phylogenetic information, species distribution modeling, and ensemble machine learning.[209] The authors showed that the functional phytochemical diversity and identity could be predicted from phylogenetic branching and ecological characteristics, offering an approach to discover bioactive molecules outside the well-established biodiversity hotspot. The association of phylogeny with phytochemical diversity suggests the advantage of combining genomics with metabolomics to identify the genes and unknown metabolite intermediates associated with specialized metabolism.

4.5. Gene cluster analysis to link the genome architect with the metabolome

Until recently, plant metabolic gene clusters were regarded as unreal, given the complexity of the genome structure and the compartmentalized biosynthesis of specialized metabolites. However, recent studies have identified the physical proximity of genes associated with specialized metabolism, highlighting the possibility of loose or partial gene clusters in plant genomes.[210] For well-characterized biosynthetic pathways of specialized metabolites, such as anthocyanins, carotenoids, and glucosinolates, genes are not clustered and are rather scattered throughout the genome. Nevertheless, with the increased number of available high-quality genomes, genome mining has shown clear evidence of clustered genes associated with different specialized metabolic pathways. The significant proximity of genes associated with specialized metabolism on the chromosomes of *Arabidopsis* has been identified.[211] Since the first report on metabolic gene clusters associated with benzoxazinoid biosynthesis in maize, over 20 clusters associated with the biosynthesis of diverse classes of specialized metabolites, including diterpenes, triterpenes, polyketides, steroidal alkaloids, monoterpene indole alkaloids, benzylisoquinoline alkaloids, and cyanogenic glycosides, have been identified and validated across different plant species.[132,133] The unexpected phenomenon of prevalent specialized metabolite gene clusters across different plant species offers a unique opportunity to discover and characterize genes and metabolites associated with specialized metabolism. One of the most remarkable gene clusters identified was in the *Opium* (poppy) genome; the noscapine gene cluster included the (*S*)- to (*R*)-reticuline (STORR) gene fusion and four genes associated with morphine alkaloid biosynthesis, representing a total of 28 genes localized in the 584 Kb region of chromosome 11.[212] The further analysis of this genome revealed that all functionally characterized BIA biosynthetic genes are part of the gene clusters, including several potential functional genes such as *PS1126530.1* (cytochrome P450) and *PS1126590.1* (methyltransferase) co-expressed with 15 other genes from the BIA biosynthetic pathway.

Recently developed toolsets such as PlantClusterFinder,[213] PhytoClust,[214] and plantiSMASH[215] allow users to select the gene segment length, co-expression pattern, similarity with previously identified plant metabolic gene clusters, number of tandem repeats, and type of member enzymes as screening criteria to predict the plant gene clusters. PhytoClust and plantiSMASH offer gene co-expression as one of the criteria to identify the gene clusters. PlantClusterFinder relies on the assigned genes to a given pathway and uses the knowledge of previously identified gene clusters to identify new gene clusters. With relatively fewer functional gene clusters being identified, whether the origin of gene clusters in plants is to provide the advantage of co-expression by shared promoter elements and the local chromatin environment, as in the case of microorganisms, remains debatable. Based on comparative genomics for known gene clusters, coinheritance has been proposed as the central driver of cluster formation.[207] Comparisons of the thalianol gene cluster at the species level revealed differences in cluster organization and auxiliary gene involvement, with the interplay between core and unlinked auxiliary genes elucidating a mechanism underlying diversification across plant species.[216] The analysis of gene clusters in the *O. pumila* genome identified 357 potential gene clusters, including 30 gene clusters associated with MIA biosynthesis,[207] conserved across plant species. Remarkably, while most gene clusters were conserved and collinear between the *O. pumila* and coffee genomes, a single gene encoding STR was lost from the functional gene cluster C1541 of the coffee genome. Further comparative genomics and phylogenetic analysis showed that retaining STR was vital for the evolution of MIA biosynthesis—an opportunity that was lost for coffee, resulting in a completely different chemotype of this species. Similarly, gene clusters associated with various other metabolic pathways are also heterogeneous, with genes in the morphine and SG pathways being scattered and genes in thebaine and noscapine pathways being closely clustered.[217] The conserved nature of the gene clusters reported thus far in the plant species producing similar specialized metabolites and the associated dynamics within the genomic region suggests gene clusters as metabolic modules for the evolution and maintenance of chemodiversity. The reduced rate of recombination between the genes at proximity explains the reason for conserved gene clusters across species.[210] Simultaneously, this could serve as a positive selection force for genes related to local adaptation. During evolution, the tandem duplication of genes within a gene cluster and sub-/neo-functionalization could offer a means to expand the metabodiversity, thus offering a site for the active evolution and expansion of chemodiversity. Conversely, the loss of critical genes or the entire gene cluster would result in a loss of the ability to retain or evolve an entire family of specialized metabolites and, ultimately, the dominance of other metabolite families to expand within the plant species.[207]

The physical proximity of gene clusters conserved across plant families does offer a case to expect the production of similar metabolite classes, as observed in the case of wide-spread localization of C-terminal *trans*-prenyltransferase and N-terminal terpene synthase involved in the biosynthesis of a large sesterterpene repertoire in *Brassicaceae*.[218] While the number of identified gene clusters across plant genomes is growing, there is no clear approach to rationalize, that is, to select genes for functional characterization, restricting one's ability to take full advantage of these discoveries. A gene cluster in which members are strongly coexpressed can be prioritized for functional characterization, and encoded enzymes can be tailored to provide clues into the prediction of new metabolites. In cases of conserved gene clusters across plant species that produce similar classes of specialized metabolites, gene information can be tailored to predict metabolite intermediates, which can be further validated using NMR or MS/MS-based approaches. Combining coexpression analysis, integrative omics, comparative genomics, and phylogenomics for gene sets assigned to a given gene cluster offers an exciting avenue for resolving the structures of some unknown mass features identified through metabolomics. This would provide a means to prioritize the mass features for further characterization and validation.

4.6. Machine learning and genomics to explore cellular components involved in specialized metabolism

The discovery of new metabolites, including their structural features, has always been a challenge. Exploiting the advantages of the increasing number of high-quality genomes and approaches such as machine learning and deep learning has the potential to drive genome-assisted natural product discovery in plants, and future efforts in this direction are warranted. Machine learning approaches rely on large datasets to avoid data overfitting. Machine learning in genomics is not new and has been at the core of gene prediction, protein domain prediction based on sequence information, and promoter motif prediction.[132,219,220] Since recent genomics analyses have generated massive data, the application of deep learning-based neural network approaches in biology has offered opportunities of using genomics to predict molecular signatures, including transcription factors, epigenetic markers, chromatin state, histone binding state, and gene expression.[219] Almost all these tools have been developed, tested, and applied to human or animal genomics, and the direct transfer for plants is possible. However, specific properties that characterize the plant genome[219] must be considered. For instance, modeling the gene expression levels for maize must consider the tetraploid nature of its genome, which would lead to the biased quantification of the gene expression, resulting in the poor quality of the test and training datasets.[221] Advances in deep learning have been successfully exploited to develop applications and tools for plant identification, species distribution modeling, weed detection, plant disease, and pest forecasts, and crop yield predication based on images, thus playing significant roles in advancing the field of plant phenology and functional trait biology.[222,223] The application of deep and machine learning for predicting miRNAs and their targets (miTAR,[224] DeepMirTar,[225] miRAW[226]), tRNA annotation (tRNA-DL[227]), polyadenylation sites (DeepPASTA[228]), transcription factor binding sites (DeFine,[229] DeepBind,[230] DeepSEA[231]), long non-coding RNAs (IncRNA-LSTM[232]), genomic methylation cites (DeepCpG[233]), RNA-binding protein binding sites (pysster[234]), protein–protein interactions (DPPI,[235] AutoCorrelation,[236] and SigProd[237]), essential genes (DeepHE[238]), and phenotype based on genotype (DeepGS[239]) underscores the potential future roles of these approaches to extract meaningful knowledge from genomic data.

One of the exciting applications of deep learning for image processing is the development of Google DeepVariant.[240] DeepVariant views mapped sequenced data as an image and treated variants as image classification to identify structural variants, including SNPs, short-indels, and inversions, and outperformed conventional mapping-based approaches in terms of accurate calling.[241] Several tools based on the DeepVariant framework have been developed, including DeepSV,[242] for the accurate identification of genomic deletions, and DeepTrio,[243] for predicting the parental structural variants, thus allowing diploid genome phasing. DeepVariant combined with other tools, particularly highly accurate long-read sequencing platforms such as PacBio, allows for the further investigation of highly repetitive regions to identify variants with roles in the evolution of desired traits and natural product biosynthetic pathways.[244] The machine- or deep learning application for predicting protein functions and tertiary structures has achieved remarkable success. For instance, the deep learning tool AlphaFold allows for the accurate prediction of protein tertiary structure based on an input gene sequence.[245,246] In this study, authors established computational method to predict protein structures *de novo* with atomic accuracy. In another related study, a three-track neural network-based integrated approach was used to transform information at the 1D sequence level, the 2D distance map level, and the 3D coordinate level to achieve relative accuracy of the predicted protein structure as achieved by AlphaFold approach.[247] Using this approach, the authors managed to generate accurate protein–protein complex models from sequence information alone, which offers key insights into the protein function of unknown structures. Compared with years of hard work put into accurately predicting protein structures using a combination of X-ray crystallography and cryo-imaging, the computational prediction of the protein structure is much faster with comparable accuracy, highlighting the possibility of predicting the functions of unknown enzymes in the future. High-quality and high-throughput prediction of enzyme commission (EC) numbers using DeepEC[248] and DEEPre[249] is another important approach in genomics-based metabolomics. Accurate EC number prediction allows to identify enzyme catalytic functions and establish gene–protein interaction relationships. Therefore, these tools are the key for building genome-scale metabolic networks and designing novel metabolic pathways. Recently, Moore *et al.* described a machine learning approach to evaluate the features associated with genes involved in specialized metabolism.[250] Although established and tested for *Arabidopsis*, the model can be transferred to other plant species for predicting genes involved in natural product biosynthesis.

The extraction of accurate genomic features is valuable for building a genome-scale metabolic model, representing a template for describing the overall ongoing metabolic processes. Modeling approaches, such as metabolism with gene expression (*i.e.*, ME model), biochemical systems approach, and kinetic modeling, are widely used, together with careful manual curation to reconstruct a metabolic model.[251,252] Genome-scale metabolic models rely on accurate gene prediction and functional annotation; therefore, advances in accurate gene prediction and annotation are valuable for expanding the scope of metabolic networks in plant natural product discovery. Genome-scale metabolic models have been successfully created for several plant species, including *Arabidopsis*, maize, oilseed rape, rice, soybean, and crassulacean acid metabolism (CAM) plants, as well as for *Chlamydomonas*.[132,251,253–255] Annotation and manual curation of metabolic network resources such as plant metabolic network (PMN),[213,256] which includes the reference database PlantCyc (https://plantcyc.org/) and 126 species/taxon-specific databases, are valuable for providing an overview of possible metabolic processes across different plant species based on a standard metabolic framework adopted from MetaCyc. For instance, PlantCyc provides access to manually curated and/or computationally predicted information on enzymes, biochemical reactions, and processes shared among or unique to over 500 plant species. Seaver *et al.* established an algorithm to streamline automated plant genome annotation and used a curated template of metabolite compartmentalization for over 100 metabolic subsystems to reconstruct metabolic models for 39 plant species.[255,257] Using this PlantSEED network, authors reconstructed plant primary metabolic model with improved compartmentalization and comparative consistency. Although the presence of a gene is not sufficient to predict whether it is functional, the annotation-based genome-scale model offers a framework to further refine metabolic models using mass balance as constraints for variables such as conditional or time-based gene expression and metabolic flux analysis, among others, resulting in reconstructed metabolic models for the accurate prediction of the metabolic state under a given condition.[251,258] Advances in predicting gene expression using gene sequences, phylogenic considerations, and chromatin state using machine learning approaches could be used to generate datasets that may serve as constraints to further refine the metabolic models. The scope of machine learning for gene feature annotation and multi-omics analysis, together with constraint-based metabolic modeling, is vast and could drive future discoveries of new metabolites.[132,258,259]

The annotation and prediction of gene function and its associated features are imperative, and much effort has been put into this area. Adopting profile-hidden Markov models (pHMMs) with homology-based gene annotation enables reliable representation based on conserved functional subunits of proteins and accurate gene functional annotation.[219] The second and third critical assessment of functional annotation (CAFA), a timed challenge to assess the computational methods for automatically assigning protein functions, has shown progress in accurately predicting molecular functionals and biological annotations, which is encouraging for future applications of genomics to detect new metabolites.[260] As the number of sequenced plant genomes is increasing and the sequencing of complex genomes is becoming more accessible, comparative genomics and phylogenetics-based identification of phytochemodiversity hotspots is feasible.[209] Integrative omics has been the core of plant functional genomics efforts to date, and by incorporating comparative genomics, a multicriteria-based approach can be used to prioritize genes and unknown metabolites for in-depth characterization.[261,262] Applying deep learning-based models to predict system responses using multi-omics datasets has seen tremendous progress; nevertheless, it is difficult to interpret these models. More interpretable models can be built using alternates such as SHAP[263] and DeepLIFT,[264] which assign importance or contribution values to the final model outcome. Future advances in accurate constraint-based genome-scale metabolic models for specialized metabolism and their integration with deep learning models would offer more avenues to discover and predict unknown metabolites.

5. Prospects of metabolomics

So far, we have discussed in detail the widely used LC-MS/MS and genomics approach based on cutting-edge techniques in metabolomics. In the following sections, several advanced and emerging technologies for metabolite annotation using other fragmentation techniques, ion mobility, and mass spectrometry imaging are highlighted. Moreover, we discuss the perspective of metabolomics and elaborate on how this area can be expanded with advances such as "virtual metabolomics" and how the AI research meets with mass spectrometry data in metabolomics to accelerate the discovery of unknown metabolites and the increase in annotation confidence.

5.1. Structure elucidation using other mass fragmentation technologies

Low-energy CID (<100 eV)-based mass fragmentation is the gold standard of LC-MS/MS-based metabolomics to elucidate the structure of metabolites. However, the MS/MS spectra do not contain all the structural information since some of the weakest chemical bonds in a molecular ion are preferentially cleaved by the accumulation of the collision energy (*i.e.*, excitation of molecular vibrations). Therefore, alternative approaches are required to complement CID-centric MS/MS spectral information and further elucidate unknown metabolite structures. Recently, several conventional and practical methodologies to determine double bond positions, *cis* (*Z*)/*trans* (*E*) isomers, and acyl chain positional isomers have become popular in lipidomics because of their biological relevance. In addition to classic ozone-induced dissociation (OzID),[265] which requires prolonged reaction time for fragmentation, the Paterno–Buchi (PB) reaction is used to determine the position of the double bonds in free fatty acids and complex lipids, such as glycero(phospho)lipids.[266,267] Following the lipid double bond reaction with acetone or 2-acetylpyridine under UV irradiation, the produced oxetane (four-membered ring ether) moiety is preferentially fragmented by low-energy CID. Combined with 2-acetylpyridine and MS^n analysis, the PB reaction can be used to determine the double bond positions and *sn*-positional isomers.[268] mCPBA epoxidation for lipid double-bond identification (MELDI)[269] can be used together with LC-MS/MS and DESI-MSI. In addition, radical-induced dissociation techniques, such as oxygen attachment dissociation (OAD),[270] electron impact excitation of ions from organics (EIEIO),[271] and ultraviolet photodissociation (UVPD),[272] are used for the same purposes but require no derivatization/reaction. In OAD, gas-phase hydroxyl radicals are introduced into the collision cell, and this radical binds to the double bond moiety, leading to odd electron-centric double bond-specific cleavage as charge remote fragmentation. EIEIO combined with EID using electron energy of 0–20 eV has been proposed, which can generate sequential fragment ions, as acquired in EI-MS. UVPD is used together with MS^n fragmentation, in which one of the fragment ions from low-energy CID is isolated and irradiated. All the above techniques can be used in combination with ion mobility separation and IMS, although the required computational support is not as adequate at the moment. Although these approaches are currently applied only in lipidomics and proteomics, they may be useful in natural product chemistry to elucidate complex molecular structures and accelerate the discovery of new molecules.

5.2. Spatial metabolomics with ion mobility (IM) spectrometry and toward spatial multi-omics

IM spectrometry has become a popular technique in metabolomics and lipidomics to increase (1) the peak capacity in MS data, (2) the purity of the MS/MS spectra, and (3) the confidence in metabolite annotation. IM spectrometry coupled with tandem MS, such as parallel accumulation-serial fragmentation (PASEF),[273] is also an attractive approach to increase the reliability of metabolite annotations. Microbial lipidome has been illustrated using LC-IM-MS/MS.[57] IM can be maximized with mass spectrometry imaging (MSI) since IM complements MSI in terms of isobaric separation and annotation confidence. Therefore, spatial metabolomics with MALDI-IM-MS[274] is expected to become popular in the coming future. Although the accuracy criteria for metabolite annotation in MSI have been reported without CCS information,[275] false discovery rate (FDR)-controlled spatial metabolomics analysis can be performed in combination with comprehensive CCS databases. In the future, data-independent MS/MS acquisition techniques may be coupled to MALDI-IM-MS to further accelerate spatial omics; therefore, CompMS tools such as METASPACE (https://metaspace2020.eu/) supporting a common data format (imzML)[276] should be developed to accelerate spatial metabolomics research.

In the coming decade, spatial multi-omics is expected to become a challenging research area of molecular biology. Various biotechniques for spatial transcriptomics have been actively developed. For instance, using Visium,[277,278] Slide-seqV2,[279] or photo-isolation chemistry (PIC),[280] RNA expression can be determined at a spatial resolution of 10–200 μm. In addition, spatial proteomics is executable using mass cytometry[281] and DNA-tagged antibody sequencing,[282] among other techniques.[283] In addition, even in spatial metabolomics, the existence of “purinosome metabolon”, which is a molecule reactor machine consisting of multiple enzymes for purine biosynthesis in a single cell, has been discovered at a spatial resolution of <1 μm using gas cluster ion beam secondary ion MS (GCIB-SIMS).[284] Advances in the relevant informatics field can further support these biotechniques. Importantly, the information on metabolite, protein, and RNA localization facilitates the study of molecular mechanisms and elucidation of plant metabolic pathways.

5.3. Plant kingdom-wide annotation

The development of MS technologies has enabled us to obtain large-scale metabolomics data with extraordinary sensitivity and accuracy at high resolution and in great detail. A certain number of metabolites in *Arabidopsis thaliana* are biosynthesized *via* roughly 27 416 genes (https://www.arabidopsis.org/index.jsp) in its genome. These metabolites show organ, tissue, and cell specificity. This spatial metabolite diversity suggests the diverse roles of metabolites in different tissues. Artificial intelligence-based approaches are expected to allow rapid and accurate chemical categorization of unknown metabolites. Biosynthetic genes of the assigned metabolites can be narrowed down by a combination of other omics approaches with metabolomics, and their functions can be revealed using genome-editing approaches, such as CRISPR-Cas9.[285] The comparative analyses of these transgenic plants lacking metabolites, as a result of genome editing, can reveal the physiological roles of these metabolites.

The next step is to expand these analyses beyond a single species. Other omics studies have shown the direction, in which metabolomics should head, *i.e.*, plant kingdom-wide annotation. There are approximately 391 000 vascular plants on the Earth,[1] and they likely produce as many metabolites as there are stars in our galaxy, earning the moniker "metabolite cosmos". To accomplish this plant kingdom-wide annotation, the performance of metabolomics must be improved in terms of chemical annotation. A key to this improvement is to add additional dimensions to the MS/MS data, such as the data acquired using SIL, isotopic fine structure analysis, and IM spectrometry. Another key is to focus on highly segmented structures (*e.g.*, functional moieties or partial structures). The strategies for N- and S-metabolites indicate that such approaches of profiling do not rely on structures but simply on the elements. Further segmentation can promote the identification of unknown metabolites with double or triple bonds; ketones; hydroxyl, amino, and carboxylic moieties; and halogens. Focusing on segmented moieties can also promote the elucidation of the complete structure based on the MS/MS spectra. However, approaches to segment the reconstruction are inadequate. Using accumulated MS/MS spectra, fragmentation patterns can be observed in certain structures. The construction of a partial structure, which can be searched in databases, will prove to be a breakthrough in finding novel metabolites.[286,287]

5.4. Virtual platform-based annotation

Two foremost problems in metabolomics are (1) the difficulty of chemical annotation and (2) the cost of instruments and software. While the former problem has been gradually solved, as described above, the latter remains unsolved in academia. To solve these problems, database or platform-based projects are ongoing. These efforts have achieved outstanding results, advancing the field of metabolomics. However, researchers still must perform chemical annotation manually. A solution is to virtually share the whole metabolome data with complete chemical annotation. Although there is a risk to the providers, it is worth actively conducting metabolomics. The providers must stipulate evidence and reference to detect incorrect annotations. Once the data are obtained from a database, researchers can perform "virtual metabolomics" using free academic software programs, such as MS-DIAL, MS-FINDER, GNPS, or currently available analytical tools. All data, free software/programs, and annotation results render access to metabolomics easier when new metabolites are explored. To reduce data, most data can be centered at particular organizations that have the required infrastructure or can reliably perform chemical annotation based on data procured from companies. An NCBI-like resource for metabolome is highly desired to promote new discoveries. Nonetheless, owing to its sheer vastness, we cannot expect to deal with phytochemodiversity even with sophisticated infrastructures, technologies, and approaches.

5.5. Artificial intelligence for mass spectrometry data (AIMS) research

Currently, 1.5 million spectral records of over 40 000 metabolites are available as the CID-MS/MS training dataset; the statistics are based on the databases explained in the section "3.2.2. Mass spectral databases for annotation". Moreover, 0.7 million records of over 570 000 compounds are available as the EI-MS training dataset; the statistics are based on the spectral records of MassBank,[32] MoNA, NIST20, and Wiley10. Given that the machine learning of face recognition has been carried out by 0.1 M–300 M face images of 1 K to 4 M identities,[288] where K and M denote kilo and million, respectively, we believe that the size of the mass spectral records is not small for machine learning research. For example, a simple question in AIMS research is to ask whether the highly accurate prediction for the existence of hydroxy moiety (–OH) is possible or not by the MS properties. Because water loss is observed in >50% molecules containing the hydroxyl moiety (see Section 3.2.3.), the accuracy would be increased when multiple properties are considered. Likewise, the prediction model for each of the 881 PubChem fingerprints and others can be constructed. Moreover, the predicted fingerprints can be used to search the structures for unknown spectra, and the idea is firstly implemented in CSI:FingerID.[289] Although the fragmentation tree (FT)[290] and kernel support vector machine (KSVM) have been used as the model parameters and machine learner, respectively, in CSI:FingerID, further AIMS research would be needed to facilitate annotation in metabolomics.

In lipidomics, the *in silico* tandem mass spectral libraries or rule-based annotations are used for the annotation pipeline,[57] in which the information of the product ion abundances are not considered, although the ion abundances can be used to predict the *sn1*/*sn2* positional isomers.[291] Because the *m*/*z* values (qualitative information) can be theoretically generated *in silico*, the next step in lipidomics annotation is to consider and predict the intensities: deep learning studies have been performed in shotgun proteomics.[292] In this light, the AIMS research for natural products is not enough yet because even the *m*/*z* values are unpredictable although the issue has been tackled by the developers of CFM-ID.[121] The important note in this area is that the number of informatics researchers in metabolomics is very small compared to that in the genomics research field. The data for AIMS researches are available, as mentioned above. Moreover, over 50 TB of raw MS data are available at Metabolomics Workbench[293] and MetaboLights[294] as the test dataset. Further stimulation of this AIMS research field will undoubtedly contribute to an increase in the accuracy and precision for predicting the molecular formula, metabolite class, substructure, molecular backbone, and even the stereochemistry of structures.

6. Conclusion

Over two decades have passed since Oliver *et al.* first used the term "metabolome" in their article.[295] Advances in metabolomics have changed plant biochemical approaches from empirical (bottom-up) to computational (top-down) methods. At present, various protocols for metabolome analysis have been proposed based on empirical experiences, and reliable computational approaches for sample preparation and MS analysis have been well established. In omics research (metabolomics, lipidomics, glycomics, proteomics, transcriptomics, and genomics), where massive data are generated every day, reproducibility, reusability, and transparency have become fundamental. Recently, many journals have encouraged to submit raw MS data (primary data) in respective repositories[293,294] and metabolite profile data (secondary data) generated from these raw data, which is a welcome step to accelerate open science.

We believe that in the coming era of computational (metabolomics) science, it will be pivotal to extract metabolites and their biological information from large-scale data with high accuracy to facilitate the reuse of metabolomics datasets and efficiently elucidate the molecular mechanisms driving the observed phenotypes. Metabolomics and the complementary techniques described in Fig. 1 require computational sciences and/or novel approaches to improve the annotation rate and to deepen the understanding of metabolisms. In addition to the role of storage of mass spectral records, the supports of fragment ion curation, spectra search engine, and knowledge conversion from chemical to biology (metabolite) will maximize the value of level 1 identification of metabolites. Moreover, processing tools, databases, and repositories for mass spectrometry imaging (MSI) should be further developed since the MSI spatial data of newly identified metabolites offer information on metabolite localization, thereby enhancing the discovery of the associated genes and proteins that are active in the same environment. Moreover, functional genomics, single cell metabolomics and transcriptomics, and spatial multi-omics techniques such as MALDI/DESI-MS for spatial metabolomics, and Visium,[277] Slide-seqV2,[279] or PIC[280] for spatial transcriptomics should be integrated efficiently with the hypothesis generated by metabolomics to mine the candidate genes that regulate novel metabolites and/or elucidate the association of the metabolites with the plant phenotype.

Moreover, increasing the annotation rate by computational sciences in combination with ion mobility, spectral library, stable isotope labeling, and ultra-high-resolution MS will enhance the discovery in metabolite-based genome-wide association study (mGWAS)[296,297] and illuminate the diversity of metabolites in the plant kingdom. Although the advances in genomics enable one to discover the gene clusters and to decode the plant revolution from the viewpoint of metabolisms, the information of metabolomics is essential not only to validate the gene functions but also to offer new opportunities for genome mining that meets with novel metabolites. In this context, computer-assisted smart metabolite annotation of data is warranted to advance the research in natural product chemistry, elucidate the true diversity of plant specialized metabolites, and identify novel drug targets. Though we have introduced a number of available spectral records and MS raw data, these numbers are still growing. Importantly, computational technologies are advancing at an astounding pace. Given the indispensability of metabolomics in biology, we hope that the present review will be helpful not only to the researchers active in this field but also to students and/or beginners undertaking metabolomics research.

7. Author contributions

K. S. initiated the project, and H. T. and R. N. coordinated the writing of this review. H. T., A. R., and R. N. wrote the parts of computational mass spectrometry, functional genomics, and cutting-edge metabolomics technique, respectively. All authors thoroughly discussed this project and helped to improve the manuscript.

8. Conflicts of interest

There are no conflicts to declare.

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10. Notes and references

1 M. Vellend, L. Baeten, A. Becker-Scarpitta, V. Boucher-Lalonde, J. L. McCune, J. Messier, I. H. Myers-Smith and D. F. Sax, *Annu. Rev. Plant Biol.*, 2017, **68**, 563–586.

2 S. Bernardini, A. Tiezzi, V. Laghezza Masci and E. Ovidi, *Nat. Prod. Res.*, 2018, **32**, 1926–1950.

3 X. M. Wu and R. X. Tan, *Nat. Prod. Rep.*, 2019, **36**, 788–809.

4 F. M. Afendi, T. Okada, M. Yamazaki, A. Hirai-Morita, Y. Nakamura, K. Nakamura, S. Ikeda, H. Takahashi, M. Altaf-Ul-Amin, L. K. Darusman, K. Saito and S. Kanaya, *Plant Cell Physiol.*, 2012, **53**, e1.

5 M. R. Wilson, L. Zha and E. P. Balskus, *J. Biol. Chem.*, 2017, **292**, 8546–8552.

6 B. Mayo, L. Vazquez and A. B. Florez, *Nutrients*, 2019, **11**, 2231.

7 D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2016, **79**, 629–661.

8 *Market reports by MarketsandMarkets, Plant Extracts Market – Forecast to 2026*, https://www.marketsandmarkets.com/Market-Reports/plant-extracts-market-942.html.

9 T. Isah, *Biol. Res.*, 2019, **52**, 39.

10 M. Erb and D. J. Kliebenstein, *Plant Physiol.*, 2020, **184**, 39–52.

11 M. M. Rinschen, J. Ivanisevic, M. Giera and G. Siuzdak, *Nat. Rev. Mol. Cell Biol.*, 2019, **20**, 353–367.

12 C. H. Johnson, J. Ivanisevic and G. Siuzdak, *Nat. Rev. Mol. Cell Biol.*, 2016, **17**, 451–459.

13 P. Trivedi, J. E. Leach, S. G. Tringe, T. Sa and B. K. Singh, *Nat. Rev. Microbiol.*, 2020, **18**, 607–621.

14 I. N. Jamil, J. Remali, K. A. Azizan, N. A. Nor Muhammad, M. Arita, H. H. Goh and W. M. Aizat, *Front. Plant Sci.*, 2020, **11**, 944.

15 K. Yugi, H. Kubota, A. Hatano and S. Kuroda, *Trends Biotechnol.*, 2016, **34**, 276–290.

16 Y. Wang, S. Y. Liu, Y. J. Hu, P. Li and J. B. Wan, *RSC Adv.*, 2015, **5**, 78728–78737.

17 A. P. Bowman, G. T. Blakney, C. L. Hendrickson, S. R. Ellis, R. M. A. Heeren and D. F. Smith, *Anal. Chem.*, 2020, **92**, 3133–3142.

18 T. Kind, H. Tsugawa, T. Cajka, Y. Ma, Z. J. Lai, S. S. Mehta, G. Wohlgemuth, D. K. Barupal, M. R. Showalter, M. Arita and O. Fiehn, *Mass Spectrom. Rev.*, 2018, **37**, 513–532.

19 J. C. Xue, C. Guijas, H. P. Benton, B. Warth and G. Siuzdak, *Nat. Methods*, 2020, **17**, 953–954.

20 Q. F. Zhu, T. Y. Zhang, L. L. Qin, X. M. Li, S. J. Zheng and Y. Q. Feng, *Anal. Chem.*, 2019, **91**, 6057–6063.

21 S. J. Zheng, S. J. Liu, Q. F. Zhu, N. Guo, Y. L. Wang, B. F. Yuan and Y. Q. Feng, *Anal. Chem.*, 2018, **90**, 8412–8420.

22 Z. Zhou, M. Luo, X. Chen, Y. Yin, X. Xiong, R. Wang and Z. J. Zhu, *Nat. Commun.*, 2020, **11**, 4334.

23 K. Giles, J. Ujma, J. Wildgoose, S. Pringle, K. Richardson, D. Langridge and M. Green, *Anal. Chem.*, 2019, **91**, 8564–8573.

24 A. C. Schrimpe-Rutledge, S. D. Sherrod and J. A. McLean, *Curr. Opin. Chem. Biol.*, 2018, **42**, 160–166.

25 J. L. Wolfender, J. M. Nuzillard, J. J. J. van der Hooft, J. H. Renault and S. Bertrand, *Anal. Chem.*, 2019, **91**, 704–742.

26 H. Tsugawa, *Curr. Opin. Biotechnol.*, 2018, **54**, 10–17.

27 K. Uppal, D. I. Walker, K. Liu, S. Z. Li, Y. M. Go and D. P. Jones, *Chem. Res. Toxicol.*, 2016, **29**, 1956–1975.

28 J. J. J. van Der Hooft, H. Mohimani, A. Bauermeister, P. C. Dorrestein, K. R. Duncan and M. H. Medema, *Chem. Soc. Rev.*, 2020, **49**, 3297–3314.

29 L. W. Sumner, A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden and M. R. Viant, *Metabolomics*, 2007, **3**, 211–221.

30 E. L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H. P. Singer and J. Hollender, *Environ. Sci. Technol.*, 2014, **48**, 2097–2098.

31 R. A. Spicer, R. Salek and C. Steinbeck, *Sci. Data*, 2017, **4**, 170138.

32 H. Horai, M. Arita, S. Kanaya, Y. Nihei, T. Ikeda, K. Suwa, Y. Ojima, K. Tanaka, S. Tanaka, K. Aoshima, Y. Oda, Y. Kakazu, M. Kusano, T. Tohge, F. Matsuda, Y. Sawada, M. Y. Hirai, H. Nakanishi, K. Ikeda, N. Akimoto, T. Maoka, H. Takahashi, T. Ara, N. Sakurai, H. Suzuki, D. Shibata, S. Neumann, T. Iida, K. Tanaka, K. Funatsu, F. Matsuura, T. Soga, R. Taguchi, K. Saito and T. Nishioka, *J. Mass Spectrom.*, 2010, **45**, 703–714.

33 M. X. Wang, J. J. Carver, V. V. Phelan, L. M. Sanchez, N. Garg, Y. Peng, D. D. Nguyen, J. Watrous, C. A. Kapono, T. Luzzatto-Knaan, C. Porto, A. Bouslimani, A. V. Melnik, M. J. Meehan, W. T. Liu, M. Criisemann, P. D. Boudreau, E. Esquenazi, M. Sandoval-Calderon, R. D. Kersten, L. A. Pace, R. A. Quinn, K. R. Duncan, C. C. Hsu, D. J. Floros, R. G. Gavilan, K. Kleigrewe, T. Northen, R. J. Dutton, D. Parrot, E. E. Carlson, B. Aigle, C. F. Michelsen, L. Jelsbak, C. Sohlenkamp, P. Pevzner, A. Edlund, J. McLean, J. Piel, B. T. Murphy, L. Gerwick, C. C. Liaw, Y. L. Yang, H. U. Humpf, M. Maansson, R. A. Keyzers, A. C. Sims, A. R. Johnson, A. M. Sidebottom, B. E. Sedio, A. Klitgaard, C. B. Larson, C. A. Boya, D. Torres-Mendoza, D. J. Gonzalez, D. B. Silva, L. M. Marques, D. P. Demarque, E. Pociute, E. C. O'Neill, E. Briand, E. J. N. Helfrich, E. A. Granatosky, E. Glukhov, F. Ryffel, H. Houson, H. Mohimani, J. J. Kharbush, Y. Zeng, J. A. Vorholt, K. L. Kurita, P. Charusanti, K. L. McPhail, K. F. Nielsen, L. Vuong, M. Elfeki, M. F. Traxler, N. Engene, N. Koyama, O. B. Vining, R. Baric, R. R. Silva, S. J. Mascuch, S. Tomasi, S. Jenkins, V. Macherla, T. Hoffman, V. Agarwal, P. G. Williams, J. Q. Dai, R. Neupane, J. Gurr, A. M. C. Rodriguez, A. Lamsa, C. Zhang, K. Dorrestein, B. M. Duggan, J. Almaliti, P. M. Allard, P. Phapale, L. F. Nothias, T. Alexandrovr, M. Litaudon, J. L. Wolfender, J. E. Kyle, T. O. Metz, T. Peryea, D. T. Nguyen, D. VanLeer, P. Shinn, A. Jadhav, R. Muller, K. M. Waters, W. Y. Shi, X. T. Liu, L. X. Zhang, R. Knight, P. R. Jensen, B. O. Palsson, K. Pogliano, R. G. Linington, M. Gutierrez, N. P. Lopes, W. H. Gerwick, B. S. Moore, P. C. Dorrestein and N. Bandeira, *Nat. Biotechnol.*, 2016, **34**, 828–837.

34 F. Qiu, D. D. Fine, D. J. Wherritt, Z. Lei and L. W. Sumner, *Anal. Chem.*, 2016, **88**, 11373–11383.

35 K. Morreel, Y. Saeys, O. Dima, F. Lu, Y. Van de Peer, R. Vanholme, J. Ralph, B. Vanholme and W. Boerjan, *Plant Cell*, 2014, **26**, 929–945.

36 J. J. van der Hooft, J. Wandy, M. P. Barrett, K. E. Burgess and S. Rogers, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 13738–13743.

37 H. Mohimani, A. Gurevich, A. Shlemov, A. Mikheenko, A. Korobeynikov, L. Cao, E. Shcherbin, L. F. Nothias, P. C. Dorrestein and P. A. Pevzner, *Nat. Commun.*, 2018, **9**, 4035.

38 T. Ngo, A. V. Ilatovskiy, A. G. Stewart, J. L. J. Coleman, F. M. McRobb, R. P. Riek, R. M. Graham, R. Abagyan, I. Kufareva and N. J. Smith, *Nat. Chem. Biol.*, 2021, **17**, 501.

39 R. Nakabayashi, M. Kusano, M. Kobayashi, T. Tohge, K. Yonekura-Sakakibara, N. Kogure, M. Yamazaki, M. Kitajima, K. Saito and H. Takayama, *Phytochemistry*, 2009, **70**, 1017–1029.

40 Z. G. Yang, R. Nakabayashi, Y. Okazaki, T. Mori, S. Takamatsu, S. Kitanaka, J. Kikuchi and K. Saito, *Metabolomics*, 2014, **10**, 543–555.

41 T. Tohge, R. Wendenburg, H. Ishihara, R. Nakabayashi, M. Watanabe, R. Sulpice, R. Hoefgen, H. Takayama, K. Saito, M. Stitt and A. R. Fernie, *Nat. Commun.*, 2016, **7**, 12399.

42 R. M. Boiteau, D. W. Hoyt, C. D. Nicora, H. A. Kinmonth-Schultz, J. K. Ward and K. Bingol, *Metabolites*, 2018, **8**, 8.

43 J. J. van der Hooft, M. Akermi, F. Y. Unlu, V. Mihaleva, V. G. Roldan, R. J. Bino, R. C. de Vos and J. Vervoort, *J. Agric. Food Chem.*, 2012, **60**, 8841–8850.

44 A. Bhatia, S. J. Sarma, Z. Lei and L. W. Sumner, *Methods Mol. Biol.*, 2019, **2037**, 113–133.

45 K. Yonekura-Sakakibara, T. Tohge, F. Matsuda, R. Nakabayashi, H. Takayama, R. Niida, A. Watanabe-Takahashi, E. Inoue and K. Saito, *Plant Cell*, 2008, **20**, 2160–2176.

46 K. Scheubert, F. Hufsky and S. Bocker, *J. Cheminf.*, 2013, **5**, 12.

47 R. R. da Silva, P. C. Dorrestein and R. A. Quinn, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 12549–12550.

48 P. M. Seitzer and B. C. Searle, *J. Proteome Res.*, 2019, **18**, 791–796.

49 A. T. Aron, E. C. Gentry, K. L. McPhail, L. F. Nothias, M. Nothias-Esposito, A. Bouslimani, D. Petras, J. M. Gauglitz, N. Sikora, F. Vargas, J. J. J. van Der Hooft, M. Ernst, K. Bin Kang, C. M. Aceves, A. M. Caraballo-Rodriguez, I. Koester, K. C. Weldon, S. Bertrand, C. Roullier, K. Y. Sun, R. M. Tehan, C. A. Boya, M. H. Christian, M. Gutierrez, A. M. Ulloa, J. A. T. Mora, R. Mojica-Flores, J. Lakey-Beitia, V. Vasquez-Chaves, Y. Zhang, A. I. Calderon, N. Tayler, R. A. Keyzers, F. Tugizimana, N. Ndlovu, A. A. Aksenov, A. K. Jarmusch, R. Schmid, A. W. Truman, N. Bandeira, M. X. Wang and P. C. Dorrestein, *Nat. Protoc.*, 2020, **15**, 1954–1991.

50 J. Xia and D. S. Wishart, *Nucleic Acids Res.*, 2010, **38**, W71–W77.

51 M. R. Molenaar, A. Jeucken, T. A. Wassenaar, C. H. A. van de Lest, J. F. Brouwers and J. B. Helms, *Gigascience*, 2019, **8**, giz061.

52 K. McLuskey, J. Wandy, I. Vincent, J. J. J. van der Hooft, S. Rogers, K. Burgess and R. Daly, *Metabolites*, 2021, **11**, 103.

53 R. Nakabayashi and K. Saito, *Curr. Opin. Plant Biol.*, 2020, **55**, 84–92.

54 R. Nakabayashi, T. Mori, N. Takeda, K. Toyooka, H. Sudo, H. Tsugawa and K. Saito, *Anal. Chem.*, 2020, **92**, 5670–5675.

55 H. Tsugawa, T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst, O. Fiehn and M. Arita, *Nat. Methods*, 2015, **12**, 523–526.

56 H. Tsugawa, R. Nakabayashi, T. Mori, Y. Yamada, M. Takahashi, A. Rai, R. Sugiyama, H. Yamamoto, T. Nakaya, M. Yamazaki, R. Kooke, J. A. Bac-Molenaar, N. Oztolan-Erol, J. J. B. Keurentjes, M. Arita and K. Saito, *Nat. Methods*, 2019, **16**, 295–298.

57 H. Tsugawa, K. Ikeda, M. Takahashi, A. Satoh, Y. Mori, H. Uchino, N. Okahashi, Y. Yamada, I. Tada, P. Bonini, Y. Higashi, Y. Okazaki, Z. W. Zhou, Z. J. Zhu, J. Koelmel, T. Cajka, O. Fiehn, K. Saito, M. Arita and M. Arita, *Nat. Biotechnol.*, 2020, **38**, 1159–1163.

58 X. Huang, Y. J. Chen, K. Cho, I. Nikolskiy, P. A. Crawford and G. J. Patti, *Anal. Chem.*, 2014, **86**, 1632–1639.

59 C. Bueschl, B. Kluger, N. K. N. Neumann, M. Doppler, V. Maschietto, G. G. Thallinger, J. Meng-Reiterer, R. Krska and R. Schuhmacher, *Anal. Chem.*, 2017, **89**, 9518–9526.

60 R. Nakabayashi, Y. Sawada, Y. Yamada, M. Suzuki, M. Y. Hirai, T. Sakurai and K. Saito, *Anal. Chem.*, 2013, **85**, 1310–1315.

61 C. J. Thompson, M. Witt, S. Forcisi, F. Moritz, N. Kessler, F. H. Laukien and P. Schmitt-Kopplin, *J. Am. Soc. Mass Spectrom.*, 2020, **31**, 2025–2034.

62 R. Nakabayashi, K. Hashimoto, K. Toyooka and K. Saito, *Anal. Chem.*, 2017, **89**, 2698–2703.

63 R. Nakabayashi and K. Saito, *Curr. Opin. Biotechnol.*, 2017, **43**, 8–16.

64 R. Nakabayashi, Z. G. Yang, T. Nishizawa, T. Mori and K. Saito, *J. Nat. Prod.*, 2015, **78**, 1179–1183.

65 P. M. Kumara, R. U. Shaanker and T. Pradeep, *Phytochemistry*, 2019, **159**, 20–29.

66 Y. H. Dong, P. Sonawane, H. Cohen, G. Polturak, L. Feldberg, S. H. Avivi, I. Rogachev and A. Aharoni, *New Phytol.*, 2020, **228**, 1986–2002.

67 R. Nakabayashi, K. Hashimoto, K. Toyooka and K. Saito, *Metabolomics*, 2019, **15**, 24.

68 S. Giacomello, F. Salmen, B. K. Terebieniec, S. Vickovic, J. F. Navarro, A. Alexeyenko, J. Reimegard, L. S. McKee, C. Mannapperuma, V. Bulone, P. L. Stahl, J. F. Sundstrom, N. R. Street and J. Lundeberg, *Nat. Plants*, 2017, **3**, 17061.

69 Y. Shinozaki, P. Nicolas, N. Fernandez-Pozo, Q. Y. Ma, D. J. Evanich, Y. N. Shi, Y. M. Xu, Y. Zheng, S. I. Snyder, L. B. B. Martin, E. Ruiz-May, T. W. Thannhauser, K. S. Chen, D. S. Domozych, C. Catala, Z. J. Fei, L. A. Mueller, J. J. Giovannoni and J. K. C. Rose, *Nat. Commun.*, 2018, **9**, 364.

70 S. Giacomello and J. Lundeberg, *Nat. Protoc.*, 2018, **13**, 2425–2446.

71 Y. H. Liu, S. Lu, K. F. Liu, S. Wang, L. Q. Huang and L. P. Guo, *Plant Methods*, 2019, **15**, 135.

72 T. Fujii, S. Matsuda, M. L. Tejedor, T. Esaki, I. Sakane, H. Mizuno, N. Tsuyama and T. Masujima, *Nat. Protoc.*, 2015, **10**, 1445–1456.

73 T. Nakashima, H. Wada, S. Morita, R. Erra-Balsells, K. Hiraoka and H. Nonami, *Anal. Chem.*, 2016, **88**, 3049–3057.

74 K. Yamamoto, K. Takahashi, H. Mizuno, A. Anegawa, K. Ishizaki, H. Fukaki, M. Ohnishi, M. Yamazaki, T. Masujima and T. Mimura, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 3891–3896.

75 K. Yamamoto, K. Takahashi, L. Caputi, H. Mizuno, C. E. Rodriguez-Lopez, T. Iwasaki, K. Ishizaki, H. Fukaki, M. Ohnishi, M. Yamazaki, T. Masujima, S. E. O'Connor and T. Mimura, *New Phytol.*, 2019, **224**, 848–859.

76 C. Rich-Griffin, A. Stechemesser, J. Finch, E. Lucas, S. Ott and P. Schafer, *Trends Plant Sci.*, 2020, **25**, 186–197.

77 R. Spicer, R. M. Salek, P. Moreno, D. Canueto and C. Steinbeck, *Metabolomics*, 2017, **13**, 106.

78 H. Y. Chang, S. M. Colby, X. Du, J. D. Gomez, M. J. Helf, K. Kechris, C. R. Kirkpatrick, S. Li, G. J. Patti, R. S. Renslow, S. Subramaniam, M. Verma, J. Xia and J. D. Young, *Anal. Chem.*, 2021, **93**, 1912–1923.

79 Z. J. Lai, H. Tsugawa, G. Wohlgemuth, S. Mehta, M. Mueller, Y. X. Zheng, A. Ogiwara, J. Meissen, M. Showalter, K. Takeuchi, T. Kind, P. Beal, M. Arita and O. Fiehn, *Nat. Methods*, 2018, **15**, 53–56.

80 C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan and G. Siuzdak, *Anal. Chem.*, 2006, **78**, 779–787.

81 R. Tautenhahn, G. J. Patti, D. Rinehart and G. Siuzdak, *Anal. Chem.*, 2012, **84**, 5035–5039.

82 T. Pluskal, S. Castillo, A. Villar-Briones and M. Oresic, *BMC Bioinf.*, 2010, **11**, 395.

83 H. L. Rost, T. Sachsenberg, S. Aiche, C. Bielow, H. Weisser, F. Aicheler, S. Andreotti, H. C. Ehrlich, P. Gutenbrunner, E. Kenar, X. Liang, S. Nahnsen, L. Nilse, J. Pfeuffer, G. Rosenberger, M. Rurik, U. Schmitt, J. Veit, M. Walzer, D. Wojnar, W. E. Wolski, O. Schilling, J. S. Choudhary, L. Malmstrom, R. Aebersold, K. Reinert and O. Kohlbacher, *Nat. Methods*, 2016, **13**, 741–748.

84 J. Chong, O. Soufan, C. Li, I. Caraus, S. Z. Li, G. Bourque, D. S. Wishart and J. G. Xia, *Nucleic Acids Res.*, 2018, **46**, W486–W494.

85 H. Tsugawa, T. Kind, R. Nakabayashi, D. Yukihira, W. Tanaka, T. Cajka, K. Saito, O. Fiehn and M. Arita, *Anal. Chem.*, 2016, **88**, 7946–7958.

86 O. Fraisier-Vannier, J. Chervin, G. Cabanac, V. Puech, S. Fournier, V. Durand, A. Amiel, O. Andre, O. A. Benamar, B. Dumas, H. Tsugawa and G. Marti, *Anal. Chem.*, 2020, **92**, 9971–9981.

87 B. C. DeFelice, S. S. Mehta, S. Samra, T. Cajka, B. Wancewicz, J. F. Fahrmann and O. Fiehn, *Anal. Chem.*, 2017, **89**, 3250–3255.

88 X. Shen, R. Wang, X. Xiong, Y. Yin, Y. Cai, Z. Ma, N. Liu and Z. J. Zhu, *Nat. Commun.*, 2019, **10**, 1516.

89 H. Treutler, H. Tsugawa, A. Porzel, K. Gorzolka, A. Tissier, S. Neumann and G. U. Balcke, *Anal. Chem.*, 2016, **88**, 8082–8090.

90 A. Smirnov, Y. Qiu, W. Jia, D. I. Walker, D. P. Jones and X. Du, *Anal. Chem.*, 2019, **91**, 9069–9077.

91 M. Wang, A. K. Jarmusch, F. Vargas, A. A. Aksenov, J. M. Gauglitz, K. Weldon, D. Petras, R. da Silva, R. Quinn, A. V. Melnik, J. J. J. van der Hooft, A. M. Caraballo-Rodriguez, L. F. Nothias, C. M. Aceves, M. Panitchpakdi, E. Brown, F. Di Ottavio, N. Sikora, E. O. Elijah, L. Labarta-Bajo, E. C. Gentry, S. Shalapour, K. E. Kyle, S. P. Puckett, J. D. Watrous, C. S. Carpenter, A. Bouslimani, M. Ernst, A. D. Swafford, E. I. Zuniga, M. J. Balunas, J. L. Klassen, R. Loomba, R. Knight, N. Bandeira and P. C. Dorrestein, *Nat. Biotechnol.*, 2020, **38**, 23–26.

92 A. K. Jarmusch, M. Wang, C. M. Aceves, R. S. Advani, S. Aguirre, A. A. Aksenov, G. Aleti, A. T. Aron, A. Bauermeister, S. Bolleddu, A. Bouslimani, A. M. Caraballo Rodriguez, R. Chaar, R. Coras, E. O. Elijah, M. Ernst, J. M. Gauglitz, E. C. Gentry, M. Husband, S. A. Jarmusch, K. L. Jones 2nd, Z. Kamenik, A. Le Gouellec, A. Lu, L. I. McCall, K. L. McPhail, M. J. Meehan, A. V. Melnik, R. C. Menezes, Y. A. Montoya Giraldo, N. H. Nguyen, L. F. Nothias, M. Nothias-Esposito, M. Panitchpakdi, D. Petras, R. A. Quinn, N. Sikora, J. J. J. van der Hooft, F. Vargas, A. Vrbanac, K. C. Weldon, R. Knight, N. Bandeira and P. C. Dorrestein, *Nat. Methods*, 2020, **17**, 901–904.

93 J. J. J. van der Hooft, J. Wandy, M. P. Barrett, K. E. V. Burgess and S. Rogers, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 13738–13743.

94 Y. Sawada, R. Nakabayashi, Y. Yamada, M. Suzuki, M. Sato, A. Sakata, K. Akiyama, T. Sakurai, F. Matsuda, T. Aoki, M. Y. Hirai and K. Saito, *Phytochemistry*, 2012, **82**, 38–45.

95 I. Tada, H. Tsugawa, I. Meister, P. Zhang, R. Shu, R. Katsumi, C. E. Wheelock, M. Arita and R. Chaleckis, *Metabolites*, 2019, **9**, 251.

96 E. L. Schymanski, C. Ruttkies, M. Krauss, C. Brouard, T. Kind, K. Duhrkop, F. Allen, A. Vaniya, D. Verdegem, S. Bocker, J. Rousu, H. Shen, H. Tsugawa, T. Sajed, O. Fiehn, B. Ghesquiere and S. Neumann, *J. Cheminf.*, 2017, **9**, 22.

97 Z. T. Lei, L. Jing, F. Qiu, H. Zhang, D. Huhman, Z. Q. Zhou and L. W. Sumner, *Anal. Chem.*, 2015, **87**, 7373–7381.

98 S. Lee, S. Hwang, M. Seo, K. B. Shin, K. H. Kim, G. W. Park, J. Y. Kim, J. S. Yoo and K. T. No, *Phytochemistry*, 2020, **177**, 112427.

99 R. Aoyagi, K. Ikeda, Y. Isobe and M. Arita, *J. Lipid Res.*, 2017, **58**, 2229–2237.

100 S. R. Heller, A. McNaught, I. Pletnev, S. Stein and D. Tchekhovskoi, *J. Cheminf.*, 2015, **7**, 23.

101 D. S. Wishart, Y. D. Feunang, A. Marcu, A. C. Guo, K. Liang, R. Vazquez-Fresno, T. Sajed, D. Johnson, C. Li, N. Karu, Z. Sayeeda, E. Lo, N. Assempour, M. Berjanskii, S. Singhal, D. Arndt, Y. Liang, H. Badran, J. Grant, A. Serra-Cayuela, Y. Liu, R. Mandal, V. Neveu, A. Pon, C. Knox, M. Wilson, C. Manach and A. Scalbert, *Nucleic Acids Res.*, 2018, **46**, D608–D617.

102 A. Frolkis, C. Knox, E. Lim, T. Jewison, V. Law, D. D. Hau, P. Liu, B. Gautam, S. Ly, A. C. Guo, J. Xia, Y. Liang, S. Shrivastava and D. S. Wishart, *Nucleic Acids Res.*, 2010, **38**, D480–D487.

103 E. Fahy, S. Subramaniam, R. C. Murphy, M. Nishijima, C. R. Raetz, T. Shimizu, F. Spener, G. van Meer, M. J. Wakelam and E. A. Dennis, *J. Lipid Res.*, 2009, **50**(suppl), S9–S14.

104 T. Jewison, C. Knox, V. Neveu, Y. Djoumbou, A. C. Guo, J. Lee, P. Liu, R. Mandal, R. Krishnamurthy, I. Sinelnikov, M. Wilson and D. S. Wishart, *Nucleic Acids Res.*, 2012, **40**, D815–D820.

105 A. C. Guo, T. Jewison, M. Wilson, Y. Liu, C. Knox, Y. Djoumbou, P. Lo, R. Mandal, R. Krishnamurthy and D. S. Wishart, *Nucleic Acids Res.*, 2013, **41**, D625–D630.

106 A. Foroutan, C. Fitzsimmons, R. Mandal, H. Piri-Moghadam, J. Zheng, A. Guo, C. Li, L. L. Guan and D. S. Wishart, *Metabolites*, 2020, **10**, 233.

107 D. S. Wishart, Y. D. Feunang, A. C. Guo, E. J. Lo, A. Marcu, J. R. Grant, T. Sajed, D. Johnson, C. Li, Z. Sayeeda, N. Assempour, I. Iynkkaran, Y. F. Liu, A. Maciejewski, N. Gale, A. Wilson, L. Chin, R. Cummings, D. Le, A. Pon, C. Knox and M. Wilson, *Nucleic Acids Res.*, 2018, **46**, D1074–D1082.

108 P. Zhang, K. Dreher, A. Karthikeyan, A. Chi, A. Pujar, R. Caspi, P. Karp, V. Kirkup, M. Latendresse, C. Lee, L. A. Mueller, R. Muller and S. Y. Rhee, *Plant Physiol.*, 2010, **153**, 1479–1491.

109 J. Hastings, G. Owen, A. Dekker, M. Ennis, N. Kale, V. Muthukrishnan, S. Turner, N. Swainston, P. Mendes and C. Steinbeck, *Nucleic Acids Res.*, 2016, **44**, D1214–D1219.

110 D. Wishart, D. Arndt, A. Pon, T. Sajed, A. C. Guo, Y. Djoumbou, C. Knox, M. Wilson, Y. J. Liang, J. Grant, Y. F. Liu, S. A. Goldansaz and S. M. Rappaport, *Nucleic Acids Res.*, 2015, **43**, D928–D934.

111 D. K. Barupal and O. Fiehn, *Environ. Health Perspect.*, 2019, **127**, 97008.

112 J. A. van Santen, G. Jacob, A. L. Singh, V. Aniebok, M. J. Balunas, D. Bunsko, F. C. Neto, L. Castano-Espriu, C. Chang, T. N. Clark, J. L. C. Little, D. A. Delgadillo, P. C. Dorrestein, K. R. Duncan, J. M. Egan, M. M. Galey, F. P. J. Haeckl, A. Hua, A. H. Hughes, D. Iskakova, A. Khadilkar, J. H. Lee, S. Lee, N. LeGrow, D. Y. Liu, J. M. Macho, C. S. McCaughey, M. H. Medema, R. P. Neupane, T. J. O'Donnell, J. S. Paula, L. M. Sanchez, A. F. Shaikh, S. Soldatou, B. R. Terlouw, T. A. Tran, M. Valentine, J. J. J. van der Hooft, D. A. Vo, M. X. Wang, D. Wilson, K. E. Zink and R. G. Linington, *ACS Cent. Sci.*, 2019, **5**, 1824–1833.

113 F. Ntie-Kang, K. K. Telukunta, K. Doring, C. V. Simoben, A. M. AF, Y. I. Malange, L. E. Njume, J. N. Yong, W. Sippl and S. Gunther, *J. Nat. Prod.*, 2017, **80**, 2067–2076.

114 J. Gu, Y. Gui, L. Chen, G. Yuan, H. Z. Lu and X. Xu, *PLoS One*, 2013, **8**, e62839.

115 S. Kim, J. Chen, T. J. Cheng, A. Gindulyte, J. He, S. Q. He, Q. L. Li, B. A. Shoemaker, P. A. Thiessen, B. Yu, L. Zaslavsky, J. Zhang and E. E. Bolton, *Nucleic Acids Res.*, 2019, **47**, D1102–D1109.

116 M. Sorokina and C. Steinbeck, *J. Cheminf.*, 2020, 12.

117 Y. Djoumbou Feunang, R. Eisner, C. Knox, L. Chepelev, J. Hastings, G. Owen, E. Fahy, C. Steinbeck, S. Subramanian, E. Bolton, R. Greiner and D. S. Wishart, *J. Cheminf.*, 2016, **8**, 61.

118 M. Sorokina, P. Merseburger, K. Rajan, M. A. Yirik and C. Steinbeck, *J. Cheminf.*, 2021, **13**, 2.

119 K. Duhrkop, M. Fleischauer, M. Ludwig, A. A. Aksenov, A. V. Melnik, M. Meusel, P. C. Dorrestein, J. Rousu and S. Bocker, *Nat. Methods*, 2019, **16**, 299–302.

120 K. Duhrkop, L. F. Nothias, M. Fleischauer, R. Reher, M. Ludwig, M. A. Hoffmann, D. Petras, W. H. Gerwick, J. Rousu, P. C. Dorrestein and S. Bocker, *Nat. Biotechnol.*, 2020, **39**, 462–471.

121 Y. Djoumbou-Feunang, A. Pon, N. Karu, J. Zheng, C. Li, D. Arndt, M. Gautam, F. Allen and D. S. Wishart, *Metabolites*, 2019, **9**.

122 F. Allen, A. Pon, M. Wilson, R. Greiner and D. Wishart, *Nucleic Acids Res.*, 2014, **42**, W94–W99.

123 S. Wolf, S. Schmidt, M. Muller-Hannemann and S. Neumann, *BMC Bioinf.*, 2010, **11**, 148.

124 C. Ruttkies, E. L. Schymanski, S. Wolf, J. Hollender and S. Neumann, *J. Cheminf.*, 2016, **8**, 3.

125 L. Ridder, J. J. van der Hooft and S. Verhoeven, *Mass Spectrometry*, 2014, **3**, S0033.

126 J. J. J. van der Hooft, J. Vervoort, R. J. Bino, J. Beekwilder and R. C. H. de Vos, *Anal. Chem.*, 2011, **83**, 409–416.

127 J. A. Falkner, J. W. Falkner, A. K. Yocum and P. C. Andrews, *J. Proteome Res.*, 2008, **7**, 4614–4622.

128 J. L. Durant, B. A. Leland, D. R. Henry and J. G. Nourse, *J. Chem. Inf. Comput. Sci.*, 2002, **42**, 1273–1280.

129 E. L. Willighagen, J. W. Mayfield, J. Alvarsson, A. Berg, L. Carlsson, N. Jeliazkova, S. Kuhn, T. Pluskal, M. Rojas-Cherto, O. Spjuth, G. Torrance, C. T. Evelo, R. Guha and C. Steinbeck, *J. Cheminf.*, 2017, **9**, 33.

130 J. Klekota and F. P. Roth, *Bioinformatics*, 2008, **24**, 2518–2525.

131 S. M. Colby, D. G. Thomas, J. R. Nunez, D. J. Baxter, K. R. Glaesemann, J. M. Brown, M. A. Pirrung, N. Govind, J. G. Teeguarden, T. O. Metz and R. S. Renslow, *Anal. Chem.*, 2019, **91**, 4346–4356.

132 A. Rai, M. Yamazaki and K. Saito, *Current Opinion in Systems Biology*, 2019, **15**, 58–67.

133 A. Rai, K. Saito and M. Yamazaki, *Plant J.*, 2017, **90**, 764–787.

134 M. Yamazaki, A. Rai, N. Yoshimoto and K. Saito, *Plant Biotechnology Reports*, 2018, **12**, 69–75.

135 M. Mutwil, *Curr. Opin. Plant Biol.*, 2020, **55**, 38–46.

136 J. H. Leebens-Mack, M. S. Barker, E. J. Carpenter, M. K. Deyholos, M. A. Gitzendanner, S. W. Graham, I. Grosse, Z. Li, M. Melkonian, S. Mirarab, M. Porsch, M. Quint, S. A. Rensing, D. E. Soltis, P. S. Soltis, D. W. Stevenson, K. K. Ullrich, N. J. Wickett, L. DeGironimo, P. P. Edger, I. E. Jordon-Thaden, S. Joya, T. Liu, B. Melkonian, N. W. Miles, L. Pokorny, C. Quigley, P. Thomas, J. C. Villarreal, M. M. Augustin, M. D. Barrett, R. S. Baucom, D. J. Beerling, R. M. Benstein, E. Biffin, S. F. Brockington, D. O. Burge, J. N. Burris, K. P. Burris, V. Burtet-Sarramegna, A. L. Caicedo, S. B. Cannon, Z. Çebi, Y. Chang, C. Chater, J. M. Cheeseman, T. Chen, N. D. Clarke, H. Clayton, S. Covshoff, B. J. Crandall-Stotler, H. Cross, C. W. dePamphilis, J. P. Der, R. Determann, R. C. Dickson, V. S. Di Stilio, S. Ellis, E. Fast, N. Feja, K. J. Field, D. A. Filatov, P. M. Finnegan, S. K. Floyd, B. Fogliani, N. García, G. Gâteblé, G. T. Godden, F. Goh, S. Greiner, A. Harkess, J. M. Heaney, K. E. Helliwell, K. Heyduk, J. M. Hibberd, R. G. J. Hodel, P. M. Hollingsworth, M. T. J. Johnson, R. Jost, B. Joyce, M. V. Kapralov, E. Kazamia, E. A. Kellogg, M. A. Koch, M. Von Konrat, K. Könyves, T. M. Kutchan, V. Lam, A. Larsson, A. R. Leitch, R. Lentz, F.-W. Li, A. J. Lowe, M. Ludwig, P. S. Manos, E. Mavrodiev, M. K. McCormick, M. McKain, T. McLellan, J. R. McNeal, R. E. Miller, M. N. Nelson, Y. Peng, P. Ralph, D. Real, C. W. Riggins, M. Ruhsam, R. F. Sage, A. K. Sakai, M. Scascitella, E. E. Schilling, E.-M. Schlösser, H. Sederoff, S. Servick, E. B. Sessa, A. J. Shaw, S. W. Shaw, E. M. Sigel, C. Skema, A. G. Smith, A. Smithson, C. N. Stewart, J. R. Stinchcombe, P. Szövényi, J. A. Tate, H. Tiebel, D. Trapnell, M. Villegente, C.-N. Wang, S. G. Weller, M. Wenzel, S. Weststrand, J. H. Westwood, D. F. Whigham, S. Wu, A. S. Wulff, Y. Yang, D. Zhu, C. Zhuang, J. Zuidof, M. W. Chase, J. C. Pires, C. J. Rothfels, J. Yu, C. Chen, L. Chen, S. Cheng, J. Li, R. Li, X. Li, H. Lu, Y. Ou, X. Sun, X. Tan, J. Tang, Z. Tian, F. Wang, J. Wang, X. Wei, X. Xu, Z. Yan, F. Yang,

X. Zhong, F. Zhou, Y. Zhu, Y. Zhang, S. Ayyampalayam, T. J. Barkman, N.-p. Nguyen, N. Matasci, D. R. Nelson, E. Sayyari, E. K. Wafula, R. L. Walls, T. Warnow, H. An, N. Arrigo, A. E. Baniaga, S. Galuska, S. A. Jorgensen, T. I. Kidder, H. Kong, P. Lu-Irving, H. E. Marx, X. Qi, C. R. Reardon, B. L. Sutherland, G. P. Tiley, S. R. Welles, R. Yu, S. Zhan, L. Gramzow, G. Theißen, G. K.-S. Wong and I. One, Thousand Plant Transcriptomes, *Nature*, 2019, **574**, 679–685.

137 H. A. Lewin, G. E. Robinson, W. J. Kress, W. J. Baker, J. Coddington, K. A. Crandall, R. Durbin, S. V. Edwards, F. Forest, M. T. P. Gilbert, M. M. Goldstein, I. V. Grigoriev, K. J. Hackett, D. Haussler, E. D. Jarvis, W. E. Johnson, A. Patrinos, S. Richards, J. C. Castilla-Rubio, M. A. van Sluys, P. S. Soltis, X. Xu, H. Yang and G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 4325–4333.

138 G. Yan, H. Liu, H. Wang, Z. Lu, Y. Wang, D. Mullan, J. Hamblin and C. Liu, *Front. Plant Sci.*, 2017, **8**, 1786.

139 A. Habib, J. J. Powell, J. Stiller, M. Liu, S. Shabala, M. Zhou, D. M. Gardiner and C. Liu, *Theor. Appl. Genet.*, 2018, **131**, 613–624.

140 A. J. Monforte and S. D. Tanksley, *Genome*, 2000, **43**, 803–813.

141 K. W. Broman, *Genetics*, 2005, **169**, 1133–1146.

142 D. Balakrishnan, M. Surapaneni, V. R. Yadavalli, K. R. Addanki, S. Mesapogu, K. Beerelli and S. Neelamraju, *Sci. Rep.*, 2020, **10**, 7766.

143 Z. H. Sun, J. Du, X. Y. Pu, M. K. Ali, X. M. Yang, C. L. Duan, M. R. Ren, X. Li and Y. W. Zeng, *Agronomy*, 2019, **9**, 40.

144 M. L. Ali, P. L. Sanchez, S. B. Yu, M. Lorieux and G. C. Eizenga, *Rice*, 2010, **3**, 218–234.

145 D. Balakrishnan, M. Surapaneni, S. Mesapogu and S. Neelamraju, *Theor. Appl. Genet.*, 2019, **132**, 1–25.

146 X. Li, W. Wang, Z. Wang, K. Li, Y. P. Lim and Z. Piao, *Front. Plant Sci.*, 2015, **6**, 432.

147 H. J. Klee and D. M. Tieman, *Nat. Rev. Genet.*, 2018, **19**, 347–356.

148 M. S. Mia, H. Liu, X. Wang and G. Yan, *Front. Plant Sci.*, 2019, **10**, 271.

149 R. Z. Yuan, N. Zhao, B. Usman, L. Luo, S. Y. Liao, Y. F. Qin, G. Nawaz and R. B. Li, *Genes*, 2020, **11**, 980.

150 S. Alseekh, T. Tohge, R. Wendenberg, F. Scossa, N. Omranian, J. Li, S. Kleessen, P. Giavalisco, T. Pleban, B. Mueller-Roeber, D. Zamir, Z. Nikoloski and A. R. Fernie, *Plant Cell*, 2015, **27**, 485–512.

151 D. Jaganathan, A. Bohra, M. Thudi and R. K. Varshney, *Theor. Appl. Genet.*, 2020, **133**, 1791–1810.

152 M. Kusano, Z. G. Yang, Y. Okazaki, R. Nakabayashi, A. Fukushima and K. Saito, *Mol. Plant*, 2015, **8**, 58–67.

153 D. Wang, W. Sun, Z. Yuan, Q. Sun, K. Fan, C. Zhang and S. Yu, *Sci. Rep.*, 2021, **11**, 189.

154 F. Matsuda, Y. Okazaki, A. Oikawa, M. Kusano, R. Nakabayashi, J. Kikuchi, J. I. Yonemaru, K. Ebana, M. Yano and K. Saito, *Plant J.*, 2012, **70**, 624–636.

155 L. Gong, W. Chen, Y. Q. Gao, X. Q. Liu, H. Y. Zhang, C. G. Xu, S. B. Yu, Q. F. Zhang and J. Luo, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 20320–20325.

156 K. Li, D. H. Wang, L. Gong, Y. Y. Lyu, H. Guo, W. Chen, C. Jin, X. Q. Liu, C. Y. Fang and J. Luo, *Plant J.*, 2019, **100**, 908–922.

157 G. H. Xu, J. J. Cao, X. F. Wang, Q. Y. Chen, W. W. Jin, Z. Li and F. Tian, *Plant Cell*, 2019, **31**, 1990–2009.

158 D. Knoch, D. Riewe, R. C. Meyer, A. Boudichevskaia, R. Schmidt and T. Altmann, *J. Exp. Bot.*, 2017, **68**, 1655–1667.

159 A. Schilmiller, F. Shi, J. Kim, A. L. Charbonneau, D. Holmes, A. Daniel Jones and R. L. Last, *Plant J.*, 2010, **62**, 391–403.

160 A. L. Schilmiller, A. L. Charbonneau and R. L. Last, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 16377–16382.

161 S. Alseekh, I. Ofner, Z. Liu, S. Osorio, J. Vallarino, R. L. Last, D. Zamir, T. Tohge and A. R. Fernie, *Plant J.*, 2020, **103**, 2007–2024.

162 G. Zhu, S. Wang, Z. Huang, S. Zhang, Q. Liao, C. Zhang, T. Lin, M. Qin, M. Peng, C. Yang, X. Cao, X. Han, X. Wang, E. van der Knaap, Z. Zhang, X. Cui, H. Klee, A. R. Fernie, J. Luo and S. Huang, *Cell*, 2018, **172**, 249–261 e212.

163 J. Szymanski, S. Bocobza, S. Panda, P. Sonawane, P. D. Cardenas, J. Lashbrooke, A. Kamble, N. Shahaf, S. Meir, A. Bovy, J. Beekwilder, Y. Tikunov, I. Romero de la Fuente, D. Zamir, I. Rogachev and A. Aharoni, *Nat. Genet.*, 2020, **52**, 1111–1121.

164 T. T. Shi, A. N. Zhu, J. Q. Jia, X. Hu, J. Chen, W. Liu, X. F. Ren, D. F. Sun, A. R. Fernie, F. Cui and W. Chen, *Plant J.*, 2020, **103**, 279–292.

165 N. Carreno-Quintero, A. Acharjee, C. Maliepaard, C. W. B. Bachem, R. Mumm, H. Bouwmeester, R. G. F. Visser and J. J. B. Keurentjes, *Plant Physiol.*, 2012, **158**, 1306–1318.

166 A. Maharijaya, B. Vosman, K. Pelgrom, Y. Wahyuni, R. C. H. de Vos and R. E. Voorrips, *Arthropod-Plant Interactions*, 2019, **13**, 1–9.

167 C. Addo-Quaye, E. Buescher, N. Best, V. Chaikam, I. Baxter and B. P. Dilkes, *G3: Genes, Genomes, Genet.*, 2017, **7**, 413–425.

168 C. Fang, A. R. Fernie and J. Luo, *Trends Plant Sci.*, 2019, **24**, 83–98.

169 S. D. Turner-Hissong, M. E. Mabry, T. M. Beissinger, J. Ross-Ibarra and J. C. Pires, *Curr. Opin. Plant Biol.*, 2020, **54**, 93–100.

170 D. Weigel, *Plant Physiol.*, 2012, **158**, 2–22.

171 V. D. Daygon, M. Calingacion, L. C. Forster, J. J. Voss, B. D. Schwartz, B. Ovenden, D. E. Alonso, S. R. McCouch, M. J. Garson and M. A. Fitzgerald, *Sci. Rep.*, 2017, **7**, 8767.

172 F. Matsuda, R. Nakabayashi, Z. Yang, Y. Okazaki, J. Yonemaru, K. Ebana, M. Yano and K. Saito, *Plant J.*, 2015, **81**, 13–23.

173 C. Fang and J. Luo, *Plant J.*, 2019, **97**, 91–100.

174 X. Huang and B. Han, *Annu. Rev. Plant Biol.*, 2014, **65**, 531–551.

175 W. Chen, Y. Gao, W. Xie, L. Gong, K. Lu, W. Wang, Y. Li, X. Liu, H. Zhang, H. Dong, W. Zhang, L. Zhang, S. Yu, G. Wang, X. Lian and J. Luo, *Nat. Genet.*, 2014, **46**, 714–721.

176 R. R. Fuentes, D. Chebotarov, J. Duitama, S. Smith, J. F. De la Hoz, M. Mohiyuddin, R. A. Wing, K. L. McNally, T. Tatarinova, A. Grigoriev, R. Mauleon and N. Alexandrov, *Genome Res.*, 2019, **29**, 870–880.

177 R. C. Strauch, E. Svedin, B. Dilkes, C. Chapple and X. Li, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 11726–11731.

178 C. Sauvage, V. Segura, G. Bauchet, R. Stevens, P. T. Do, Z. Nikoloski, A. R. Fernie and M. Causse, *Plant Physiol.*, 2014, **165**, 1120–1132.

179 P. D. Sonawane, A. Jozwiak, S. Panda and A. Aharoni, *Curr. Opin. Plant Biol.*, 2020, **55**, 118–128.

180 H. Sonah, L. O'Donoughue, E. Cober, I. Rajcan and F. Belzile, *Plant Biotechnol. J.*, 2015, **13**, 211–221.

181 B. Brachi, N. Faure, M. Horton, E. Flahauw, A. Vazquez, M. Nordborg, J. Bergelson, J. Cuguen and F. Roux, *PLoS Genet.*, 2010, **6**, e1000940.

182 S. Zhou, K. A. Kremling, N. Bandillo, A. Richter, Y. K. Zhang, K. R. Ahern, A. B. Artyukhin, J. X. Hui, G. C. Younkin, F. C. Schroeder, E. S. Buckler and G. Jander, *Plant Cell*, 2019, **31**, 937–955.

183 P. E. Bayer, A. A. Golicz, A. Scheben, J. Batley and D. Edwards, *Nat. Plants*, 2020, **6**, 914–920.

184 M. F. Danilevicz, C. G. Tay Fernandez, J. I. Marsh, P. E. Bayer and D. Edwards, *Curr. Opin. Plant Biol.*, 2020, **54**, 18–25.

185 C. Da Silva, G. Zamperin, A. Ferrarini, A. Minio, A. Dal Molin, L. Venturini, G. Buson, P. Tononi, C. Avanzato, E. Zago, E. Boido, E. Dellacassa, C. Gaggero, M. Pezzotti, F. Carrau and M. Delledonne, *Plant Cell*, 2013, **25**, 4777–4788.

186 L. Gao, I. Gonda, H. Sun, Q. Ma, K. Bao, D. M. Tieman, E. A. Burzynski-Chang, T. L. Fish, K. A. Stromberg, G. L. Sacks, T. W. Thannhauser, M. R. Foolad, M. J. Diez, J. Blanca, J. Canizares, Y. Xu, E. van der Knaap, S. Huang, H. J. Klee, J. J. Giovannoni and Z. Fei, *Nat. Genet.*, 2019, **51**, 1044–1051.

187 S. P. Gordon, B. Contreras-Moreira, D. P. Woods, D. L. Des Marais, D. Burgess, S. Shu, C. Stritt, A. C. Roulin, W. Schackwitz, L. Tyler, J. Martin, A. Lipzen, N. Dochy, J. Phillips, K. Barry, K. Geuten, H. Budak, T. E. Juenger, R. Amasino, A. L. Caicedo, D. Goodstein, P. Davidson, L. A. J. Mur, M. Figueroa, M. Freeling, P. Catalan and J. P. Vogel, *Nat. Commun.*, 2017, **8**, 2184.

188 G. Haberer, N. Kamal, E. Bauer, H. Gundlach, I. Fischer, M. A. Seidel, M. Spannagl, C. Marcon, A. Ruban, C. Urbany, A. Nemri, F. Hochholdinger, M. Ouzunova, A. Houben, C. C. Schon and K. F. X. Mayer, *Nat. Genet.*, 2020, **52**, 950–957.

189 Y. Liu, H. Du, P. Li, Y. Shen, H. Peng, S. Liu, G. A. Zhou, H. Zhang, Z. Liu, M. Shi, X. Huang, Y. Li, M. Zhang, Z. Wang, B. Zhu, B. Han, C. Liang and Z. Tian, *Cell*, 2020, **182**, 162–176 e113.

190 Q. Zhao, Q. Feng, H. Lu, Y. Li, A. Wang, Q. Tian, Q. Zhan, Y. Lu, L. Zhang, T. Huang, Y. Wang, D. Fan, Y. Zhao, Z. Wang, C. Zhou, J. Chen, C. Zhu, W. Li, Q. Weng, Q. Xu, Z. X. Wang, X. Wei, B. Han and X. Huang, *Nat. Genet.*, 2018, **50**, 278–284.

191 W. Wang, R. Mauleon, Z. Hu, D. Chebotarov, S. Tai, Z. Wu, M. Li, T. Zheng, R. R. Fuentes, F. Zhang, L. Mansueto, D. Copetti, M. Sanciangco, K. C. Palis, J. Xu, C. Sun, B. Fu, H. Zhang, Y. Gao, X. Zhao, F. Shen, X. Cui, H. Yu, Z. Li, M. Chen, J. Detras, Y. Zhou, X. Zhang, Y. Zhao, D. Kudrna, C. Wang, R. Li, B. Jia, J. Lu, X. He, Z. Dong, J. Xu, Y. Li, M. Wang, J. Shi, J. Li, D. Zhang, S. Lee, W. Hu, A. Poliakov, I. Dubchak, V. J. Ulat, F. N. Borja, J. R. Mendoza, J. Ali, J. Li, Q. Gao, Y. Niu, Z. Yue, M. E. B. Naredo, J. Talag, X. Wang, J. Li, X. Fang, Y. Yin, J. C. Glaszmann, J. Zhang, J. Li, R. S. Hamilton, R. A. Wing, J. Ruan, G. Zhang, C. Wei, N. Alexandrov, K. L. McNally, Z. Li and H. Leung, *Nature*, 2018, **557**, 43–49.

192 P. Zhou, K. A. Silverstein, T. Ramaraj, J. Guhlin, R. Denny, J. Liu, A. D. Farmer, K. P. Steele, R. M. Stupar, J. R. Miller, P. Tiffin, J. Mudge and N. D. Young, *BMC Genomics*, 2017, **18**, 261.

193 M. Alonge, X. Wang, M. Benoit, S. Soyk, L. Pereira, L. Zhang, H. Suresh, S. Ramakrishnan, F. Maumus, D. Ciren, Y. Levy, T. H. Harel, G. Shalev-Schlosser, Z. Amsellem, H. Razifard, A. L. Caicedo, D. M. Tieman, H. Klee, M. Kirsche, S. Aganezov, T. R. Ranallo-Benavidez, Z. H. Lemmon, J. Kim, G. Robitaille, M. Kramer, S. Goodwin, W. R. McCombie, S. Hutton, J. Van Eck, J. Gillis, Y. Eshed, F. J. Sedlazeck, E. van der Knaap, M. C. Schatz and Z. B. Lippman, *Cell*, 2020, **182**, 145–161 e123.

194 J. M. Song, Z. Guan, J. Hu, C. Guo, Z. Yang, S. Wang, D. Liu, B. Wang, S. Lu, R. Zhou, W. Z. Xie, Y. Cheng, Y. Zhang, K. Liu, Q. Y. Yang, L. L. Chen and L. Guo, *Nat. Plants*, 2020, **6**, 34–45.

195 S. Hubner, N. Bercovich, M. Todesco, J. R. Mandel, J. Odenheimer, E. Ziegler, J. S. Lee, G. J. Baute, G. L. Owens, C. J. Grassa, D. P. Ebert, K. L. Ostevik, B. T. Moyers, S. Yakimowski, R. R. Masalia, L. Gao, I. Calic, J. E. Bowers, N. C. Kane, D. Z. H. Swanevelder, T. Kubach, S. Munos, N. B. Langlade, J. M. Burke and L. H. Rieseberg, *Nat. Plants*, 2019, **5**, 54–62.

196 J. D. Montenegro, A. A. Golicz, P. E. Bayer, B. Hurgobin, H. Lee, C. K. Chan, P. Visendi, K. Lai, J. Dolezel, J. Batley and D. Edwards, *Plant J.*, 2017, **90**, 1007–1013.

197 A. A. Golicz, P. E. Bayer, G. C. Barker, P. P. Edger, H. Kim, P. A. Martinez, C. K. Chan, A. Severn-Ellis, W. R. McCombie, I. A. Parkin, A. H. Paterson, J. C. Pires, A. G. Sharpe, H. Tang, G. R. Teakle, C. D. Town, J. Batley and D. Edwards, *Nat. Commun.*, 2016, **7**, 13390.

198 G. D. Moghe and R. L. Last, *Plant Physiol.*, 2015, **169**, 1512–1523.

199 R. C. Moore and M. D. Purugganan, *Curr. Opin. Plant Biol.*, 2005, **8**, 122–128.

200 B. R. Lichman, G. T. Godden and C. R. Buell, *Curr. Opin. Plant Biol.*, 2020, **55**, 74–83.

201 Y. He, A. Galant, Q. Y. Pang, J. M. Strul, S. F. Balogun, J. M. Jez and S. X. Chen, *J. Biol. Chem.*, 2011, **286**, 28794–28801.

202 B. J. Leong and R. L. Last, *Curr. Opin. Struct. Biol.*, 2017, **47**, 105–112.

203 Y. Shimizu, A. Rai, Y. Okawa, H. Tomatsu, M. Sato, K. Kera, H. Suzuki, K. Saito and M. Yamazaki, *Plant J.*, 2019, **100**, 505–521.

204 E. Pichersky and E. Lewinsohn, *Annu. Rev. Plant Biol.*, 2011, **62**, 549–566.

205 S. Xu, T. Brockmoller, A. Navarro-Quezada, H. Kuhl, K. Gase, Z. Ling, W. Zhou, C. Kreitzer, M. Stanke, H. Tang, E. Lyons, P. Pandey, S. P. Pandey, B. Timmermann, E. Gaquerel and I. T. Baldwin, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 6133–6138.

206 R. Huang, A. J. O'Donnell, J. J. Barboline and T. J. Barkman, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 10613–10618.

207 A. Rai, H. Hirakawa, R. Nakabayashi, S. Kikuchi, K. Hayashi, M. Rai, H. Tsugawa, T. Nakaya, T. Mori, H. Nagasaki, R. Fukushi, Y. Kusuya, H. Takahashi, H. Uchiyama, A. Toyoda, S. Hikosaka, E. Goto, K. Saito and M. Yamazaki, *Nat. Commun.*, 2021, **12**, 405.

208 S. Weaver, S. D. Shank, S. J. Spielman, M. Li, S. V. Muse and S. L. Kosakovsky Pond, *Mol. Biol. Evol.*, 2018, **35**, 773–777.

209 E. Defossez, C. Pitteloud, P. Descombes, G. Glauser, P. M. Allard, T. W. N. Walker, P. Fernandez-Conradi, J. L. Wolfender, L. Pellissier and S. Rasmann, *Proc. Natl. Acad. Sci. U. S. A.*, 2021, 118.

210 H. W. Nutzmann, C. Scazzocchio and A. Osbourn, *Annu. Rev. Genet.*, 2018, **52**, 159–183.

211 J. H. Wisecaver, A. T. Borowsky, V. Tzin, G. Jander, D. J. Kliebenstein and A. Rokas, *Plant Cell*, 2017, **29**, 944–959.

212 L. Guo, T. Winzer, X. Yang, Y. Li, Z. Ning, Z. He, R. Teodor, Y. Lu, T. A. Bowser, I. A. Graham and K. Ye, *Science*, 2018, **362**, 343–347.

213 P. Schlapfer, P. Zhang, C. Wang, T. Kim, M. Banf, L. Chae, K. Dreher, A. K. Chavali, R. Nilo-Poyanco, T. Bernard, D. Kahn and S. Y. Rhee, *Plant Physiol.*, 2017, **173**, 2041–2059.

214 N. Topfer, L. M. Fuchs and A. Aharoni, *Nucleic Acids Res.*, 2017, **45**, 7049–7063.

215 S. A. Kautsar, H. G. Suarez Duran, K. Blin, A. Osbourn and M. H. Medema, *Nucleic Acids Res.*, 2017, **45**, W55–W63.

216 Z. Liu, J. Cheema, M. Vigouroux, L. Hill, J. Reed, P. Paajanen, L. Yant and A. Osbourn, *Nat. Commun.*, 2020, **11**, 5354.

217 Q. Li, S. Ramasamy, P. Singh, J. M. Hagel, S. M. Dunemann, X. Chen, R. Chen, L. Yu, J. E. Tucker, P. J. Facchini and S. Yeaman, *Nat. Commun.*, 2020, **11**, 1190.

218 A. C. Huang, S. A. Kautsar, Y. J. Hong, M. H. Medema, A. D. Bond, D. J. Tantillo and A. Osbourn, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, E6005–E6014.

219 H. Wang, E. Cimen, N. Singh and E. Buckler, *Curr. Opin. Plant Biol.*, 2020, **54**, 34–41.

220 J. Zou, M. Huss, A. Abid, P. Mohammadi, A. Torkamani and A. Telenti, *Nat. Genet.*, 2019, **51**, 12–18.

221 J. D. Washburn, M. K. Mejia-Guerra, G. Ramstein, K. A. Kremling, R. Valluru, E. S. Buckler and H. Wang, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 5542–5549.

222 S. Esposito, D. Carputo, T. Cardi and P. Tripodi, *Plants*, 2019, **9**, 34.

223 Y. Jiang and C. Li, *Plant Phenomics*, 2020, **2020**, 4152816.

224 T. Gu, X. Zhao, W. B. Barbazuk and J.-H. Lee, *BMC Bioinf.*, 2021, **22**, 96.

225 M. Wen, P. Cong, Z. Zhang, H. Lu and T. Li, *Bioinformatics*, 2018, **34**, 3781–3787.

226 A. Pla, X. Zhong and S. Rayner, *PLoS Comput. Biol.*, 2018, **14**, e1006185.

227 X. Gao, Z. Wei and H. Hakonarson, *Hum. Hered.*, 2018, **83**, 163–172.

228 A. Arefeen, X. Xiao and T. Jiang, *Bioinformatics*, 2019, **35**, 4577–4585.

229 M. Wang, C. Tai, W. E and L. Wei, *Nucleic Acids Res.*, 2018, **46**, e69.

230 B. Alipanahi, A. Delong, M. T. Weirauch and B. J. Frey, *Nat. Biotechnol.*, 2015, **33**, 831–838.

231 J. Zhou and O. G. Troyanskaya, *Nat. Methods*, 2015, **12**, 931–934.

232 J. Meng, Z. Chang, P. Zhang, W. Shi and Y. Luan, lncRNA-LSTM: Prediction of Plant Long Non-coding RNAs Using Long Short-Term Memory Based on p-nts Encoding, in *Intelligent Computing Methodologies, ICIC 2019, Lecture Notes in Computer Science*, ed. D. S. Huang, Z. K. Huang and A. Hussain, Springer, Cham, 2019, vol. 11645, DOI: 10.1007/978-3-030-26766-7_32.

233 C. Angermueller, H. J. Lee, W. Reik and O. Stegle, *Genome Biol.*, 2017, **18**, 67.

234 S. Budach and A. Marsico, *Bioinformatics*, 2018, **34**, 3035–3037.

235 S. Hashemifar, B. Neyshabur, A. A. Khan and J. Xu, *Bioinformatics*, 2018, **34**, i802–i810.

236 Y. Guo, L. Yu, Z. Wen and M. Li, *Nucleic Acids Res.*, 2008, **36**, 3025–3030.

237 S. Martin, D. Roe and J. L. Faulon, *Bioinformatics*, 2005, **21**, 218–226.

238 X. Zhang, W. Xiao and W. Xiao, *PLoS Comput. Biol.*, 2020, **16**, e1008229.

239 W. Ma, Z. Qiu, J. Song, J. Li, Q. Cheng, J. Zhai and C. Ma, *Planta*, 2018, **248**, 1307–1318.

240 R. Poplin, P. C. Chang, D. Alexander, S. Schwartz, T. Colthurst, A. Ku, D. Newburger, J. Dijamco, N. Nguyen, P. T. Afshar, S. S. Gross, L. Dorfman, C. Y. McLean and M. A. DePristo, *Nat. Biotechnol.*, 2018, **36**, 983–987.

241 T. Yun, H. Li, P. C. Chang, M. F. Lin, A. Carroll and C. Y. McLean, *Bioinformatics*, 2020, **36**, 5582–5589.

242 L. Cai, Y. Wu and J. Gao, *BMC Bioinf.*, 2019, **20**, 665.

243 E. K. K. Ip, C. Hadinata, J. W. K. Ho and E. Giannoulatou, *Bioinformatics*, 2020, **36**, 3549–3551.

244 R. Luo, F. J. Sedlazeck, T. W. Lam and M. C. Schatz, *Nat. Commun.*, 2019, **10**, 998.

245 M. AlQuraishi, *Bioinformatics*, 2019, **35**, 4862–4865.

246 J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Zidek, A. Potapenko, A. Bridgland, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman,

E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli and D. Hassabis, *Nature*, 2021, DOI: 10.1038/s41586-021-03819-2.

247 M. Baek, F. DiMaio, I. Anishchenko, J. Dauparas, S. Ovchinnikov, G. R. Lee, J. Wang, Q. Cong, L. N. Kinch, R. D. Schaeffer, C. Millán, H. Park, C. Adams, C. R. Glassman, A. DeGiovanni, J. H. Pereira, A. V. Rodrigues, A. A. van Dijk, A. C. Ebrecht, D. J. Opperman, T. Sagmeister, C. Buhlheller, T. Pavkov-Keller, M. K. Rathinaswamy, U. Dalwadi, C. K. Yip, J. E. Burke, K. C. Garcia, N. V. Grishin, P. D. Adams, R. J. Read and D. Baker, *Science*, 2021, DOI: 10.1126/science.abj8754.

248 J. Y. Ryu, H. U. Kim and S. Y. Lee, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 13996–14001.

249 Y. Li, S. Wang, R. Umarov, B. Xie, M. Fan, L. Li and X. Gao, *Bioinformatics*, 2018, **34**, 760–769.

250 B. M. Moore, P. Wang, P. Fan, B. Leong, C. A. Schenck, J. P. Lloyd, M. D. Lehti-Shiu, R. L. Last, E. Pichersky and S. H. Shiu, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 2344–2353.

251 A. Rai and K. Saito, *Curr. Opin. Biotechnol.*, 2016, **37**, 127–134.

252 L. M. Dersch, V. Beckers and C. Wittmann, *Metab. Eng.*, 2016, **34**, 1–24.

253 T. J. Clark, L. Guo, J. Morgan and J. Schwender, *Annu. Rev. Plant Biol.*, 2020, **71**, 303–326.

254 C. G. Dal'Molin, L. E. Quek, R. W. Palfreyman, S. M. Brumbley and L. K. Nielsen, *Plant Physiol.*, 2010, **154**, 1871–1885.

255 S. M. D. Seaver, C. Lerma-Ortiz, N. Conrad, A. Mikaili, A. Sreedasyam, A. D. Hanson and C. S. Henry, *Plant J.*, 2018, **95**, 1102–1113.

256 K. Dreher, *Methods Mol. Biol.*, 2014, **1083**, 151–171.

257 S. M. Seaver, S. Gerdes, O. Frelin, C. Lerma-Ortiz, L. M. Bradbury, R. Zallot, G. Hasnain, T. D. Niehaus, B. El Yacoubi, S. Pasternak, R. Olson, G. Pusch, R. Overbeek, R. Stevens, V. de Crecy-Lagard, D. Ware, A. D. Hanson and C. S. Henry, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 9645–9650.

258 G. Zampieri, S. Vijayakumar, E. Yaneske and C. Angione, *PLoS Comput. Biol.*, 2019, **15**, e1007084.

259 D. M. Camacho, K. M. Collins, R. K. Powers, J. C. Costello and J. J. Collins, *Cell*, 2018, **173**, 1581–1592.

260 N. Zhou, Y. Jiang, T. R. Bergquist, A. J. Lee, B. Z. Kacsoh, A. W. Crocker, K. A. Lewis, G. Georghiou, H. N. Nguyen, M. N. Hamid, L. Davis, T. Dogan, V. Atalay, A. S. Rifaioglu, A. Dalkiran, R. Cetin Atalay, C. Zhang, R. L. Hurto, P. L. Freddolino, Y. Zhang, P. Bhat, F. Supek, J. M. Fernandez, B. Gemovic, V. R. Perovic, R. S. Davidovic, N. Sumonja, N. Veljkovic, E. Asgari, M. R. K. Mofrad, G. Profiti, C. Savojardo, P. L. Martelli, R. Casadio, F. Boecker, H. Schoof, I. Kahanda, N. Thurlby, A. C. McHardy, A. Renaux, R. Saidi, J. Gough, A. A. Freitas, M. Antczak, F. Fabris, M. N. Wass, J. Hou, J. Cheng, Z. Wang, A. E. Romero, A. Paccanaro, H. Yang, T. Goldberg, C. Zhao, L. Holm, P. Toronen, A. J. Medlar, E. Zosa, I. Borukhov, I. Novikov, A. Wilkins, O. Lichtarge, P. H. Chi, W. C. Tseng, M. Linial, P. W. Rose, C. Dessimoz, V. Vidulin, S. Dzeroski, I. Sillitoe, S. Das, J. G. Lees, D. T. Jones, C. Wan, D. Cozzetto, R. Fa, M. Torres, A. Warwick Vesztrocy, J. M. Rodriguez, M. L. Tress, M. Frasca, M. Notaro, G. Grossi, A. Petrini, M. Re, G. Valentini, M. Mesiti, D. B. Roche, J. Reeb, D. W. Ritchie, S. Aridhi, S. Z. Alborzi, M. D. Devignes, D. C. E. Koo, R. Bonneau, V. Gligorijevic, M. Barot, H. Fang, S. Toppo, E. Lavezzo, M. Falda, M. Berselli, S. C. E. Tosatto, M. Carraro, D. Piovesan, H. Ur Rehman, Q. Mao, S. Zhang, S. Vucetic, G. S. Black, D. Jo, E. Suh, J. B. Dayton, D. J. Larsen, A. R. Omdahl, L. J. McGuffin, D. A. Brackenridge, P. C. Babbitt, J. M. Yunes, P. Fontana, F. Zhang, S. Zhu, R. You, Z. Zhang, S. Dai, S. Yao, W. Tian, R. Cao, C. Chandler, M. Amezola, D. Johnson, J. M. Chang, W. H. Liao, Y. W. Liu, S. Pascarelli, Y. Frank, R. Hoehndorf, M. Kulmanov, I. Boudellioua, G. Politano, S. Di Carlo, A. Benso, K. Hakala, F. Ginter, F. Mehryary, S. Kaewphan, J. Bjorne, H. Moen, M. E. E. Tolvanen, T. Salakoski, D. Kihara, A. Jain, T. Smuc, A. Altenhoff, A. Ben-Hur, B. Rost, S. E. Brenner, C. A. Orengo, C. J. Jeffery, G. Bosco, D. A. Hogan, M. J. Martin, C. O'Donovan, S. D. Mooney, C. S. Greene, P. Radivojac and I. Friedberg, *Genome Biol.*, 2019, **20**, 244.

261 A. Rai, M. Rai, H. Kamochi, T. Mori, R. Nakabayashi, M. Nakamura, H. Suzuki, K. Saito and M. Yamazaki, *DNA Res.*, 2020, 27.

262 L. Sun, A. Rai, M. Rai, M. Nakamura, N. Kawano, K. Yoshimatsu, H. Suzuki, N. Kawahara, K. Saito and M. Yamazaki, *J. Nat. Med.*, 2018, **72**, 867–881.

263 S. Lundberg and S. I. Lee, *Advances in Neural Information Processing Systems*, 2017, abs/1705.07874.

264 A. Shrikumar, P. Greenside and A. Kundaje, Presented in part at the Proceedings of the 34th International Conference on Machine Learning, *Proceedings of Machine Learning Research*, 2017.

265 M. C. Thomas, T. W. Mitchell, D. G. Harman, J. M. Deeley, J. R. Nealon and S. J. Blanksby, *Anal. Chem.*, 2008, **80**, 303–311.

266 J. Zhao, X. B. Xie, Q. H. Lin, X. X. Ma, P. Su and Y. Xia, *Anal. Chem.*, 2020, **92**, 13470–13477.

267 L. Chong, R. Tian, R. Shi, Z. Ouyang and Y. Xia, *Front. Chem.*, 2019, **7**, 807.

268 W. Cao, S. Cheng, J. Yang, J. Feng, W. Zhang, Z. Li, Q. Chen, Y. Xia, Z. Ouyang and X. Ma, *Nat. Commun.*, 2020, **11**, 375.

269 T. H. Kuo, H. H. Chung, H. Y. Chang, C. W. Lin, M. Y. Wang, T. L. Shen and C. C. Hsu, *Anal. Chem.*, 2019, **91**, 11905–11915.

270 H. Takahashi, Y. Shimabukuro, D. Asakawa, S. Yamauchi, S. Sekiya, S. Iwamoto, M. Wada and K. Tanaka, *Anal. Chem.*, 2018, **90**, 7230–7238.

271 T. Baba, J. L. Campbell, J. C. Y. Le Blanc, P. R. S. Baker and K. Ikeda, *J. Lipid Res.*, 2018, **59**, 910–919.

272 J. S. Brodbelt, L. J. Morrison and I. Santos, *Chem. Rev.*, 2020, **120**, 3328–3380.

273 F. Meier, A. D. Brunner, S. Koch, H. Koch, M. Lubeck, M. Krause, N. Goedecke, J. Decker, T. Kosinski, M. A. Park, N. Bache, O. Hoerning, J. Cox, O. Rather and M. Mann, *Mol. Cell. Proteomics*, 2018, **17**, 2534–2545.

274 J. Soltwisch, B. Heijs, A. Koch, S. Vens-Cappell, J. Hohndorf and K. Dreisewerd, *Anal. Chem.*, 2020, **92**, 8697–8703.

275 A. Palmer, P. Phapale, I. Chernyavsky, R. Lavigne, D. Fay, A. Tarasov, V. Kovalev, J. Fuchser, S. Nikolenko, C. Pineau, M. Becker and T. Alexandrov, *Nat. Methods*, 2017, **14**, 57–60.

276 T. Schramm, A. Hester, I. Klinkert, J. P. Both, R. M. A. Heeren, A. Brunelle, O. Laprevote, N. Desbenoit, M. F. Robbe, M. Stoeckli, B. Spengler and A. Rompp, *J. Proteomics*, 2012, **75**, 5106–5110.

277 S. Maniatis, T. Aijo, S. Vickovic, C. Braine, K. Kang, A. Mollbrink, D. Fagegaltier, Z. Andrusivova, S. Saarenpaa, G. Saiz-Castro, M. Cuevas, A. Watters, J. Lundeberg, R. Bonneau and H. Phatnani, *Science*, 2019, **364**, 89–93.

278 P. L. Stahl, F. Salmen, S. Vickovic, A. Lundmark, J. F. Navarro, J. Magnusson, S. Giacomello, M. Asp, J. O. Westholm, M. Huss, A. Mollbrink, S. Linnarsson, S. Codeluppi, A. Borg, F. Ponten, P. I. Costea, P. Sahlen, J. Mulder, O. Bergmann, J. Lundeberg and J. Frisen, *Science*, 2016, **353**, 78–82.

279 R. R. Stickels, E. Murray, P. Kumar, J. L. Li, J. L. Marshall, D. J. Di Bella, P. Arlotta, E. Z. Macosko and F. Chen, *Nat. Biotechnol.*, 2021, **39**, 313–319.

280 M. Honda, S. Oki, R. Kimura, A. Harada, K. Maehara, K. Tanaka, C. Meno and Y. Ohkawa, *Nat. Commun.*, 2021, **12**, 4416.

281 I. Paul, C. White, I. Turcinovic and A. Emili, *FEBS J.*, 2021, DOI: 10.1111/febs.15685.

282 W. Timp and G. Timp, *Sci. Adv.*, 2020, **6**, eaax8978.

283 E. Lundberg and G. H. H. Borner, *Nat. Rev. Mol. Cell Biol.*, 2019, **20**, 285–302.

284 V. Pareek, H. Tian, N. Winograd and S. J. Benkovic, *Science*, 2020, **368**, 283–290.

285 X. Liu, S. R. Wu, J. Xu, C. Sui and J. H. Wei, *Acta Pharm. Sin. B*, 2017, **7**, 292–302.

286 K. B. Kang, M. Ernst, J. J. J. van der Hooft, R. R. da Silva, J. Park, M. H. Medema, S. H. Sung and P. C. Dorrestein, *Plant J*, 2019, **98**, 1134–1144.

287 S. Rogers, C. W. Ong, J. Wandy, M. Ernst, L. Ridder and J. J. J. van der Hooft, *Faraday Discuss*, 2019, **218**, 284–302.

288 Z. Zhu, G. Huang, J. Deng, Y. Ye, J. Huang, X. Chen, J. Zhu, T. Yang, J. Lu, D. Du and J. Zhou, arXiv, 2103, 04098, 2021.

289 K. Duhrkop, H. Shen, M. Meusel, J. Rousu and S. Bocker, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 12580–12585.

290 S. Bocker and K. Duhrkop, *J. Cheminf.*, 2016, **8**, 5.

291 J. Hartler, A. Triebl, A. Ziegl, M. Trotzmuller, G. N. Rechberger, O. A. Zeleznik, K. A. Zierler, F. Torta, A. Cazenave-Gassiot, M. R. Wenk, A. Fauland, C. E. Wheelock, A. M. Armando, O. Quehenberger, Q. Zhang, M. J. O. Wakelam, G. Haemmerle, F. Spener, H. C. Kofeler and G. G. Thallinger, *Nat. Methods*, 2017, **14**, 1171–1174.

292 S. Gessulat, T. Schmidt, D. P. Zolg, P. Samaras, K. Schnatbaum, J. Zerweck, T. Knaute, J. Rechenberger, B. Delanghe, A. Huhmer, U. Reimer, H. C. Ehrlich, S. Aiche, B. Kuster and M. Wilhelm, *Nat. Methods*, 2019, **16**, 509–518.

293 M. Sud, E. Fahy, D. Cotter, K. Azam, I. Vadivelu, C. Burant, A. Edison, O. Fiehn, R. Higashi, K. S. Nair, S. Sumner and S. Subramaniam, *Nucleic Acids Res.*, 2016, **44**, D463–D470.

294 N. S. Kale, K. Haug, P. Conesa, K. Jayseelan, P. Moreno, P. Rocca-Serra, V. C. Nainala, R. A. Spicer, M. Williams, X. Li, R. M. Salek, J. L. Griffin and C. Steinbeck, *Curr. Protoc. Bioinf.*, 2016, **53**, 14 13 11–14 13 18.

295 S. G. Oliver, M. K. Winson, D. B. Kell and F. Baganz, *Trends Biotechnol.*, 1998, **16**, 373–378.

296 J. Luo, *Curr. Opin. Plant Biol.*, 2015, **24**, 31–38.

297 M. L. Slaten, A. Yobi, C. Bagaza, Y. O. Chan, V. Shrestha, S. Holden, E. Katz, C. Kanstrup, A. E. Lipka, D. J. Kliebenstein, H. H. Nour-Eldin and R. Angelovici, *Plant Physiol.*, 2020, **183**, 483–500.