

REVIEW

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# Recent developments and emerging trends of mass spectrometric methods in plant hormone analysis: a review

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## Abstract

Plant hormones are naturally occurring small molecule compounds which are present at trace amounts in plant. They play a pivotal role in the regulation of plant growth. The biological activity of plant hormones depends on their concentrations in the plant, thus, accurate determination of plant hormone is paramount. However, the complex plant matrix, wide polarity range and low concentration of plant hormones are the main hindrances to effective analyses of plant hormone even when state-of-the-art analytical techniques are employed. These factors substantially influence the accuracy of analytical results. So far, significant progress has been realized in the analysis of plant hormones, particularly in sample pretreatment techniques and mass spectrometric methods. This review describes the classic extraction and modern microextraction techniques used to analyze plant hormone. Advancements in solid phase microextraction (SPME) methods have been driven by the ever-increasing requirement for dynamic and in vivo identification of the spatial distribution of plant hormones in real-life plant samples, which would contribute greatly to the burgeoning field of plant hormone investigation. In this review, we describe advances in various aspects of mass spectrometry methods. Many fragmentation patterns are analyzed to provide the theoretical basis for the establishment of a mass spectral database for the analysis of plant hormones. We hope to provide a technical guide for further discovery of new plant hormones. More than 140 research studies on plant hormone published in the past decade are reviewed, with a particular emphasis on the recent advances in mass spectrometry and sample pretreatment techniques in the analysis of plant hormone. The potential progress for further research in plant hormones analysis is also highlighted.

**Keywords:** Plant, Plant hormone, Sample pretreatment, Mass spectrometry, Trace analysis

## Background

Plant hormones are small organic molecules that naturally occurring in plants at very low concentrations. They regulate plants germination, growth, reproduction as well as both biotic and abiotic stress responses under different environmental conditions. These molecules show diverse

chemical properties and unique chemical structures with wide polarity range and poor photo-thermal stability [1–12]. Plant hormones are categorized into several classes based on their structures and physiological functions [10, 13]. The most common classes are: auxin, cytokinin (CK), abscisic acid (ABA), gibberellin (GA), brassinosteroids (BR), salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and some newly identified plant hormones such as strigolactone (SL) [12]. Each class is defined by its physiological functions in regulating plant growth through synergistic or antagonistic [14, 15]. Therefore, simultaneous quantification of the multi-class plant hormones and

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accurate determination of spatial–temporal distribution is important to clarify the mechanisms of their recognition, regulation, and how they influence plant growth and development.

Accurate determination of plant hormones requires the development of highly sensitive and efficient analytical techniques for analysis of plant hormone. Effective analysis of plant hormones is limited by inefficient extraction procedures due to their extremely low abundance (usually at  $\text{ng g}^{-1}$  or even  $\text{pg g}^{-1}$  level) and varied concentration ranges in plants [16]. For instance, the concentration of auxin and JA in plants ranges about 1–50  $\text{ng g}^{-1}$  fresh weight (FW) whereas the content of BRs is as low as 0.01–0.1  $\text{ng g}^{-1}$  FW [17]. This calls for highly sensitive analytical strategies with a good dynamic range for quantitative analysis are required for simultaneous extraction and purification of multi-class plant hormones. Existence of several structural isomers of plant hormones also complicates the separation processes. Another factor that affects analysis of plant hormone is the matrix effect. Elimination of interference is important for obtaining accurate information on the metabolism and functions of plant hormones [18]. In addition to these features, plant hormones are unstable and extremely sensitive to the environment change such as temperature, humidity and light due to their chemical heterogeneity. For instance, GAs show high sensitivity to pH and temperature (above 40 °C) but are relatively stable under acidic conditions [19].

These challenges necessitate the development of sample pretreatment and detection methods that are highly selective, sensitive, good enrichment, ease of handling and with broader dynamic range of detection. Non-chromatographic analytical methods such as bioassays and immunoassays were widely used in the early stage of plant hormone research. In comparison with mass spectrometry (MS)-based methodologies, these methods are less sensitive, specific and cannot perform simultaneous detection of multi-class plant hormones, thus, they have been phased out with modern approaches [20]. MS detection equipped with chromatographic techniques are the most widely used analytical approaches for simultaneous analysis of multi-class plant hormones are based on MS detection equipped with chromatographic techniques. Chromatographic methods integrated with MS system provide effective analysis for multi-class plant hormones in the plant matrix (plant component except for plant hormone) [20–23]. Particularly, combining gas chromatography (GC) or liquid chromatography (LC) with different MS detectors forms a rapid, sensitive and high-throughput method for quantitative analysis of plant hormone [21, 24–26]. Furthermore, in order to simultaneous analysis multi-class plant hormones in a

single step, a derivatization step is often required before MS-based instrumental analysis because of the non-volatile and non-ionizable property of some plant hormones [27–32]. Thus, derivatization step may promotes comprehensive investigation of the distribution of plant hormone among plant tissues, and hence characterization of metabolic and signaling processes involving plant hormones [33].

MS is a promising technique for efficient analysis of plant hormones. This is because it has high detection performance [1, 11, 33–36]. For this reason, MS-based methods with better sensitivity, selectivity, accuracy and high-throughput features are used to quantitatively and qualitatively analyze plant hormone at tissue and cellular level [29, 37–39]. Given its effective mass-to-charge ( $m/z$ ) separation ability, mass analyzer provides enhanced selectivity of analytes, thereby improving quantification of trace and ultra-trace plant hormones in plant. Several types of mass analyzers have been developed such as ion trap (IT) [40], quadrupole time-of-flight (Q-TOF) [41] and triple quadrupole instruments (QQQ) [42]. Mass analyzer combined with chromatography have been extensively applied in plant hormone analysis, indicating their ability of accurate quantitative analysis and efficient separation capabilities [43]. MS-based methods can facilitate simultaneous analysis of multiple target compounds. It is therefore suitable for comprehensive analysis and elucidation of the biosynthesis, transportation, metabolic pathways, and signaling networks of plant hormones.

This review provides an up-to-date overview of modern analytical methods and sample pretreatment techniques for plant hormone analysis. Some recent applications of these methods are highlighted in this review. The fragmentation patterns of plant hormones are investigated to provide a theoretical basis for the establishment of a mass spectral database. The prospects related to the analysis of plant hormone are also proposed, which would serve as a technical guide for further discovery of new plant hormones [44, 45].

### Sample pretreatment

The key sample pretreatment strategies applied in plant hormone analysis include homogenization, extraction and purification steps (some studies incorporate a derivatization or labeling step depending on the analytical strategies and instrumental requirements) [42, 43, 46–50]. Sample pretreatment procedure is the major bottleneck that limits rapid separation and simultaneous analysis of multi-class plant hormones, especially at trace levels. To effectively extract plant hormone from complex plant samples, analytical methods that minimize matrix interferences and meet the requirements of high

sensitivity, satisfactory recovery and good reproducibility with simple operation are desirable [1, 19, 23, 36, 40, 49–52]. Previous studies have indicated that sample pretreatment is the most labor-intensive and time-consuming step in the whole analytical procedure of endogenous plant hormones [43]. In addition, sample pretreatment is the most error-prone part of the process accounting for about 30% of all sources of errors [10, 13, 18, 43]. An ideal sample pretreatment technique should be less sample consuming, exhibit high sensitivity, and display outstanding enrichment capabilities, especially during *in vivo* detection.

Accurate determination of variations in the spatial distributions of plant hormone with the aspects of *in vivo* analysis them in a trace amount of plant tissues would broaden the scope of investigating their physiological functions. The extremely low concentration of plant hormone, similarity in chemical structures and large amount of metabolites in plants are the main challenges which affect accurate detection of plant hormone as they cause interference during analyses [53]. A trace amount of sample is sufficient for investigating the content and dynamic changes of plant hormones in a specific part of the plant. This limits the use of rare and endangered plants as source of sample for analytical purposes. However, only few pretreatment methods are sensitive enough and provide high enrichment capabilities for *in vivo* analysis of trace amount of plant hormones in plant matrix [1, 51, 53–55]. On-line analysis methods provide a platform for analysis of trace compounds analysis [56]. However, due to the complexity of plant sample matrix and the low concentration of components to be measured, development of on-line techniques has been relatively very slow. In addition to *in vivo*, *in situ*, real-time and on-line analysis, sample pretreatment techniques are expected to be highly sensitive with good enrichment properties to produce reliable analytical results for plant samples with high complexity. Typical examples of sample pretreatment methods published in the recent past 5 years are shown in Table 1.

#### Common pretreatment methods in plant hormone analysis

Depending on different extraction modes, extraction method can be divided into classic extraction methods and microextraction methods. Both classic extraction and microextraction methods are based on liquid phase extraction and solid phase extraction [62]. Classic extraction methods provide near-exhaustive extraction efficiency [45]. Compared with classic extraction methods, microextraction methods have the advantages of having high extraction efficiency and miniaturization of device and enrichment capacity making them more suitable for the analysis of plant hormone [47]. The extraction

and clean-up techniques for trace compounds analysis include liquid–liquid extraction (LLE) [17, 21, 24, 30, 63–65], liquid phase microextraction (LPME) [52, 56, 66], dispersive liquid–liquid microextraction (DLLME) [40, 67], polymer monolith microextraction (PMME) [68], solid phase extraction (SPE) [26, 38, 55, 69–72], solid phase microextraction (SPME) [25, 34], magnetic solid phase extraction (MSPE) [16, 23, 73], matrix solid-phase dispersion (MSPD) [37], dispersive micro solid phase extraction (DMSPE) [74], ion pair stir bar sorptive extraction (SBSE) [1] and electromembrane extraction (EME) [75] (see Fig. 1).

The conventional LLE and SPE methods are widely used to analyze biological samples [76]. However, these sample pretreatment techniques are associated with drawbacks such as complicated operations, time-consuming, the requirement of relatively large amounts of sample and organic solvents, strong matrix effect (the interference and influence of matrix on analyte analysis) and the difficulty to automate. Flokova et al. [55] achieved rapid extraction of plant hormone with less sample consumption, less than 20 mg FW of *A. thaliana* leaf tissue in 10% methanol. They used single-step reversed-phase polymer-based SPE method which reduced the matrix effect and increased recovery of labile plant hormone.

Recent technological advancements have improved the miniaturization and automation of sample pretreatment methods. Compared with the traditional LLE and SPE methods, microextraction techniques such as DLLME, LPME, SPME and DMSPE applications in trace analysis are ever increasing. The solvent consumption, better extraction performance, minimized invasiveness and automated coupling with analytical instruments property of microextraction techniques have evidently benefited (Fig. 1) [62]. Cai et al. [77] developed a clean-up strategy employing a single-step dispersive solid-phase extraction (DSPE) combined with UPLC–MS/MS to obtain spatial–temporal information of 54 plant hormones including auxins, ABA, SA, JA, GAs and CKs. Wang et al. [75] used a combination of EME and LC–MS/MS to quantitatively detect six acidic plant hormones in 20 mg citrus leaf sample with a limit of detection (LOD) ranging from 0.1 to 10 ng mL<sup>-1</sup>. For more accuracy sample processing, sample pretreatment techniques require automation. Wang et al. [37] used an in-line coupled MSPD-MAX-MCX system to analyze a wide range of polar plant hormones in rice (200 mg). The proposed method showed higher extraction efficiency, lower matrix effect, ease of manipulation and time-saving characteristics. Recently, off-line procedures with highly efficient separation and detection sensitivity have been used to analyze plant hormone. However, the possible loss of trace plant hormone during additional process (compare with in-line procedures)

**Table 1 Representative sample pretreatment methods for the determination of plant hormone**

Analytes	Sample pretreatment <sup>a</sup>		Plant matrix	On-/off-line extraction	References
	Extraction solvent	Purification method			
BRs	80% methanol	Solid phase extraction (SPE)	<i>A. thaliana</i> leaf (10 g FW)	Off-line	[31]
IAA, ABA, JA, GAs	Acetonitrile	Sequential magnetic solid-phase extraction (MSPE)	<i>Brassica napus</i> L. flowers (100 mg FW)	Off-line	[16]
BRs	Methanol	matrix solid-phase dispersion (MSPD)	Rice (200 mg FW)	On-line	[37]
CKs, ABA, GAs, JAs, SA, BRs, SLs	2-propanol/H <sub>2</sub> O/HCl (2:1:0.002 v/v/v)	Solid phase extraction (SPE)	<i>P. pinaster</i> Aiton. clones needles (200 ± 20 mg DW)	Off-line	[54]
BRs	Acetonitrile	two-dimensional microscale solid phase extraction (2D $\mu$ SPE)	Tomato leaves (225 mg FW)	On-line	[57]
SA, IAA, ABA	Methanol	ion-pair stir bar sorptive extraction (IP-SBSE)	Cucumbers and green bean sprouts (100 mg FW)	Off-line	[1]
GAs	75% methanol, 5% formic acid	Liquid-liquid extraction (LLE)	<i>A. thaliana</i> flower	Off-line	[21]
GAs, ABA, ET, SAs, JAs, BRs	Methanol: water: HCl (6 N) (80: 19.9:0.1; v/v/v)	Liquid-liquid extraction (LLE)	Valencia sweet orange (100 ± 2 mg FW)	Off-line	[30]
SA ABA	Methanol-water-acetic acid extraction solution (80:19:1, v/v/v)	Dispersive liquid-liquid microextraction (DLLME)	Peach (250 mg FW)	Off-line	[40]
JA, IAA, SA, ABA, IBA, GA <sub>3</sub>	Centrifugation at 9500 rpm	Single-drop liquid-liquid-liquid microextraction (SD-LLLME)	Fresh fruit juice	Off-line	[58]
ABA, IAA	80% Methanol	Hollow-fiber liquid-phase micro-extraction (HF-LPME)	Soil sample (10 g)	Off-line	[59]
BRs	Acetonitrile	Polymer monolith microextraction (PMME)	Rice shoots (1 g FW leaves, or 0.5 g FW flower tissue)	Off-line	[60]
28-EpibromoBR	80% methanol	Solid-phase microextraction (SPME)	<i>A. thaliana</i> samples (400 mg FW)	On-line	[34]
NAA, 2-NOA, 2,4-D, MCPA, PAA	H <sub>2</sub> O, NaCl, HCl	Solid-phase microextraction (SPME)	Tomato (3 g FW)	Off-line	[61]

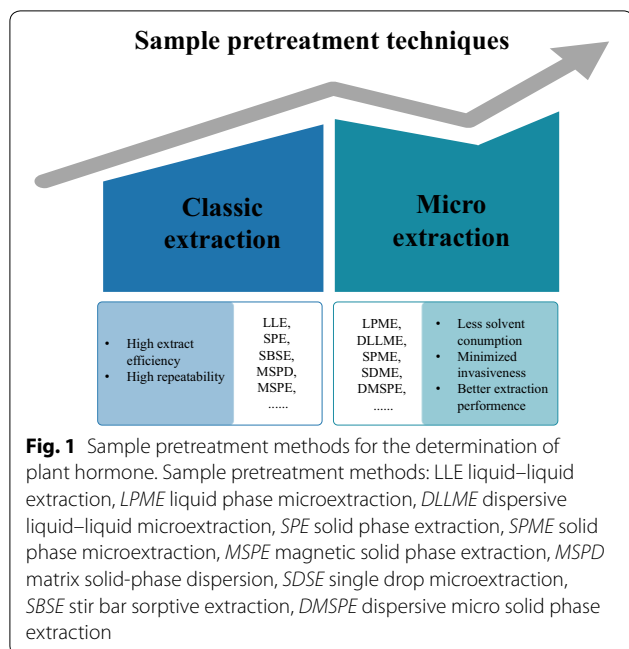
<sup>a</sup> On-line means coupling of preparation techniques, both for extraction and clean-up, and injection in a detection system; off-line means preparation techniques, both for extraction and clean-up, and injection in a detection system are carried out independently

has been an intractable challenge. Direct on-line analysis (coupling of sample preparation techniques directly to the separation and detection system) minimizes sample preparation steps and enables the effective pre-concentration and clean-up of plant samples [78]. Although these microextraction methods achieve effective plant hormone extraction, some of them are difficult to automate for on-line analysis.

### SPME strategies and advances

An ideal sample pretreatment technique for plant hormones analysis should be simple, rapid, selective, efficient, solvent-free, inexpensive, reproducible, highly accurate and avoids the degradation of analyses. For simultaneous quantitative analysis of multi-class plant hormones, the compatibility of an in vivo and automated sample pretreatment methods with dynamic and ultra-sensitive extraction capacity are required. SPME has advantages in these aspects [48, 50, 52, 79]. In SPME,

sampling, extraction, concentration, and injection are integrated into a single step, which promotes miniaturization, automation, in vivo sampling and on-line analysis [80]. However, commercialized SPME sorbents exhibit lack selectivity which affects the distribution between analytes and the stationary phase. To address this challenge, several modified SPME sorbents have been designed to improve the sensitivity and selectivity of the extraction process [81]. Liu et al. [82] used a carbowax-coated fiber as SPME sorbent hyphenated with HPLC to detect auxin. They found that the modified sorbent had a relatively much higher extraction efficiency compared to polyacrylate fibers. Its dynamic range spanned over three orders of magnitude. The LOD/limit of quantification (LOQ) values of the target compounds in pure water were 0.149 (0.497), 0.442 (1.472), 0.121 (0.403) and 0.058 (0.193)  $\mu\text{g L}^{-1}$  for IAA, ABA, IBA and NAA, respectively. Song et al. [61] applied the SPME method incorporating on-cyclodextrin (-CD)-modified carbon



nanotubes (CNTs) and a hollow fiber (HF) to analyze 1-naphthaleneacetic acid (NAA) and 2-naphthoxyacetic acid (2-NOA) in vegetables. This strategy allowed easy handling and provided environmentally friendly features as it minimized the amount of solvents. In most cases, plant hormones in plant tissues are determined simultaneously by combining SPME and HPLC [62, 76, 78]. Wang et al. [34] developed an automated and sensitive method to analyze the endogenous 28-epihomobrassinolide (28-epihomoBR) in *A. thaliana* by coupling on-line SPME with a polymer monolithic column and LC–MS (SPME-LC–MS). The poly (methacrylic acid-co-ethylene dimethacrylate) (poly (MAA-co-EDMA)) monolith was prepared in the capillary. The proposed SPME had satisfactory recovery (80.3–92.1%) and reproducibility (RSD 6.8–9.6%). Thus, SPME notably reduces assay duration and the difficulty of automation and improves *in vivo* analysis.

The application of SPME for *in vivo* analysis not only simplifies the extraction process, but also has the advantages of non-destructive extraction and easy to couple with mass spectrometry. Thus, this method facilitates on-site sampling, pretreatment and the detection of unstable analytes, making it suitable for long-term monitoring of signaling processes of plant hormones.

### Mass spectrometric analysis

Co-extraction of lipids and other interfering compounds is often eliminated by applying enrichment and purification methods. However simultaneous and accurate determination of individual or multi-class plant hormones in

plant samples is still a great challenge due to the complex plant matrix [26, 80, 81, 83]. Various strategies including biological approaches (e.g. bioassays and immunoassays) physical and chemical analytical methods have been proposed to overcome the matrix effect of plant samples during the quantification of plant hormones [18]. Despite the high sensitivity of these analytical methods, most of them are not effective enough for the simultaneous analysis of multi-class plant hormones. MS-based technologies overcome these limitations. Successful exploitation of MS-based technologies for determining plant hormone from plant tissue satisfy the needs of high sensitivity. Furthermore, MS-based technologies can be combined with different chromatographic methods, which make it possible for high throughput analysis of plant samples [32, 55, 61, 75].

Plant hormone in plants were analyzed by GC–MS initially, in which the separation was dependent on using different partition coefficients between the gas phase and the stationary phase [20, 26, 56, 84–86]. However, most plant hormones are non-volatile, therefore a derivatization step is integral, not only it converts the non-volatile plant hormones to more volatile and thermally stable derivatives, but also for changing chromatographic behavior, improving detection sensitivity and selectivity of the analytes [87]. Based on the non-volatility property of most plant hormones, liquid chromatography has high separation performance when analyzing non-volatile compounds without the necessity of derivatization, which reduces possible loss of the target compound during derivatization procedure.

### LC–MS analysis

LC is by far the most common analytical technique used to qualitatively and quantitatively analyze trace plant hormone due to its high separation performance, high detection sensitivity, fast analytical speed and easy operation [60]. LC hyphenated with MS detection provides higher sensitivity and accuracy for the simultaneous analysis of multi-class plant hormone, which promotes research on the molecular biology of plant hormones [88]. Different LC–MS techniques used to analyze plant hormone are shown in Table 2.

The ion source allows large, non-volatile molecules to be analyzed directly from the liquid phase, which ionizes the neutral atom or molecule and fragment the ion beam generated from it [93]. Two of the frequently used ion sources in plant hormone analysis are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is preferred by most scientists as it has well balanced ionization efficiency for various classes of chemical compounds and is a soft ionization method [94]. It is, therefore, suitable for analysis of polar



**Table 2 Overview of the mass spectrometry applications currently employed in plant hormone analysis**

Analytes	Analytical technique	Plant matrix	Recovery (%) <sup>a</sup>	LODs	Linearity	References
JA, ABA, SA, BA, GAs	LC-ESI-MS/MS	Hamlin trees leave (20 mg FW)	34.6–50.3	0.03–3.00 ng mL <sup>-1</sup>	0.10–100.00 ng mL <sup>-1</sup>	[75]
JAs, SA, ABA, IAA	UHPLC-ESI-MS/MS	<i>A. thaliana</i> leaf (20 mg FW)	–	0.05–50 fmol	0.05–500.00 pmol	[55]
IAA, GAs, tZ, ABA	LC-ESI-IT-MS/MS	<i>A. thaliana</i> (100 mg FW)	70.0–100.0	0.55–170 fmol	5.00–1000.00 fmol	[63]
BRs	UHPLC-MS/MS	<i>Brassica napus</i> (50 mg FW)	30.9–88.9	1–50 pg	0.01–10.00 pmol	[20]
K, iP, BA, ABA, NAA	HPLC-MS/MS	Vermicompost (250 mg)	0.3–18.9	0.0015–0.3000 mg L <sup>-1</sup>	0.005–10.000 mg L <sup>-1</sup>	[14]
IAA, ABA, GAs, SA, JA, tZ, 6-BA, iP	LC-ESI-MS/MS	Oilseed rape	75.1–113.0	0.002–0.021 ng mL <sup>-1</sup>	0.0013–0.0210 ng mL <sup>-1</sup>	[89]
tZ, K, KR	UHPLC-ESI-MS/MS	Tobacco (100 mg FW)	68.8–103.0	2.4–47 pg mL <sup>-1</sup>	0.005–20.000 ng mL <sup>-1</sup>	[90]
ABA, IAA, JA, SA, GAs, tZ	UHPLC-ESI-MS/MS	<i>P. pinaster</i> Aiton. clones needles (200 ± 20 mg DW)	56.8–99.1	1.45–23.44 pg	0.040–2500.000 ng mL <sup>-1</sup>	[54]
BRs	UHPLC-ESI-MS/MS	<i>A. thaliana</i> (10 g FW)	76.9–86.1	2.00–8.00 ng L <sup>-1</sup>	10.00–10,000.00 ng L <sup>-1</sup>	[31]
GAs	UHPLC-ESI-MS/MS	Floral organs (about 80–250 µg)	64.0–107.0	Down to 5.41 amol	0.01–25 fmol	[21]
IAA, ABA, JA, SA, IBA, GAs	CE-ESI-TOF-MS	Rice leaves (3 g)	84.6–112.2	0.34–4.59 ng mL <sup>-1</sup>	1.30–850.00 ng mL <sup>-1</sup>	[91]
IAA, ABA, JA, SA, IBA, GAs	nano-LC-ESI-QTOF-MS	Rice leaves (5 mg FW)	88.3–104.3	1.05–122.4 pg mL <sup>-1</sup>	0.004–100.00 ng mL <sup>-1</sup>	[52]
ABA, IAA, IBA, GAs, SA	HPLC-ESI-QTOF-MS	Green seaweeds (100 mg FW)	80.0–92.0	0.5–1.0 µg mL <sup>-1</sup>	0.20–100.00 mg mL <sup>-1</sup>	[92]

<sup>a</sup> The recovery is relative recovery

compounds because it does not require the rapid vaporization of solvents and ions can be generated in solution [95]. Since most plant hormones are polar in nature, ESI is comparatively a more suitable ion source for the characterization, identification and quantification of plant hormone in plant tissue [96].

#### Ionization efficiency of plant hormone analysis

The effectiveness of ionization method greatly influences the sensitivity of LC-MS analysis. Factors affecting the efficiency of ESI process includes composition and flow rate of mobile phase as well as matrix complexity of the plant sample [97]. The purification step of plant sample is crucial to improving the ionization efficiency, accuracy and sensitivity of LC-ESI-MS/MS analysis as it removes interfering co-eluting compounds which cause ion-suppression when using ESI, hence, affecting the ionization efficiency of plant hormone [98]. It is also important to optimize the composition, type and concentration of mobile phase and the purity of sample to ensure effective ionization during LC-ESI-MS analysis [99, 100]. Na<sup>+</sup>, K<sup>+</sup> and NH<sub>4</sub><sup>+</sup> are commonly present in the solvent throughout the transportation or storage processes, which are classical additives and buffers of LC.

Some mobile phase additives such as formic acid, acetic acid, trifluoroacetic acid are usually used to improve the ionization efficiency during MS detection. The chemical properties, concentration, and pH of the mobile phase additive have a significant effect on the LC-MS response [60, 87]. The type of appropriate additive added in mobile phase is critical to improve the chromatographic resolution, peak shape and ionization efficiency. Formic acid or acetic acid are the commonly used additives in the mobile phase to detect basic plant hormones in positive ion mode, whereas ammonium formate is used as the additive of the mobile phase to detect the acidic plant hormones in negative ion mode [101].

ESI is an ionization technique that converts ions in solution to gas-phase for MS analysis. The first step of ESI is the electrophoretic migration of anion and cation in the solution under an electric field to produce ions [102]. The efficiency of ion production depends on conductivity, which relies on the concentration of electrolyte. Suitable solvents for ESI include polar and moderately polar solvents and the most frequently used solvents are methanol, acetonitrile and water. Composition of the mobile phase for plant hormone analysis is usually water and acetonitrile (ACN) or methanol with suitable additives

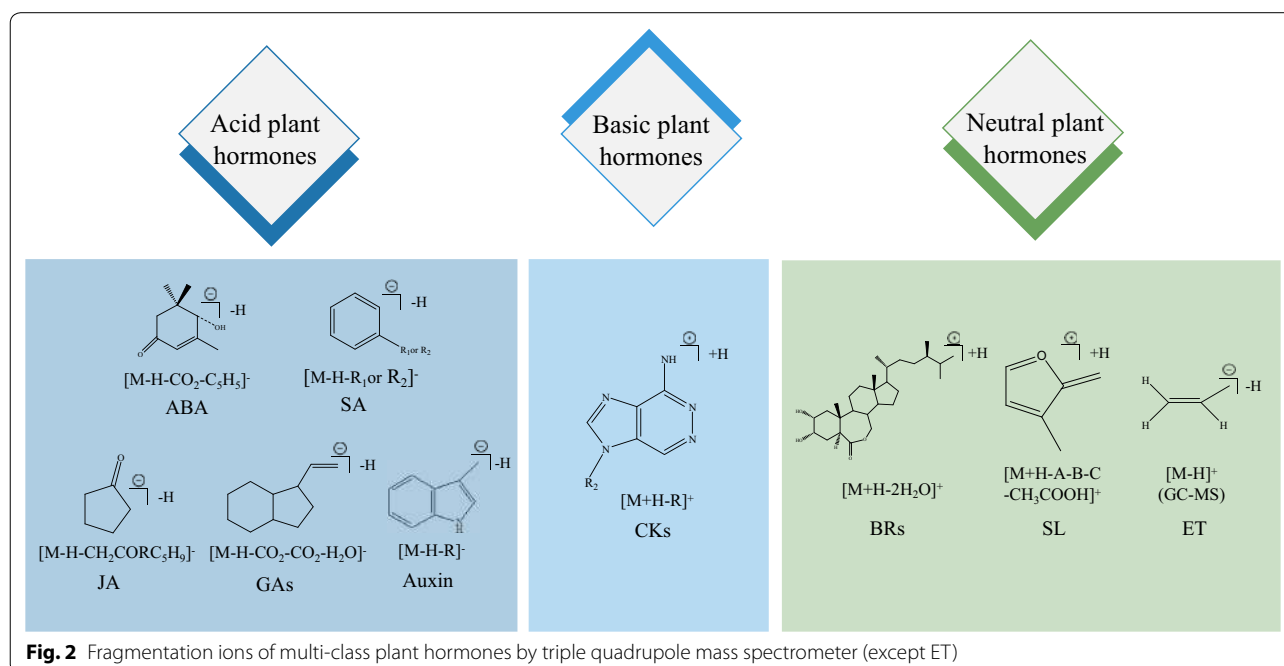
[103]. Methanol is an appropriate solvent for ESI–MS analysis of acidic compounds in the negative ionization mode. The response of some analytes in negative ESI–MS can be improved by applying methanol. In contrast, ACN is more suitable for analyzing in positive ion mode as it is an aprotic solvent [103]. The flow rate of mobile phase in the ESI ion source also influences the ionization process. Normally, smaller initial droplet is observed in the lower flow rate which decreases the number of fission cycles and the amount of solvents to be evaporated to form gas-phase ions, thus improving the electrospray effect [104].

Given the above-mentioned factors that affect the ionization efficiency, where the composition of the mobile phase affects the LC procedure. The LC analysis of plant hormone is often performed by combining water and methanol or acetonitrile with the addition of formic acid or ammonium formate as the mobile phase. The flow rate of mobile phase is often optimized at 0.2–0.5 mL min<sup>-1</sup>. Cai et al. [73] successfully separated and analyzed 8 CKs, IAA, ABA, JA and 10 GAs using a 27-min gradient of 0.05% FA H<sub>2</sub>O (A) and ACN (B) at a flow rate of 0.4 mL min<sup>-1</sup>. Suh et al. [75] characterized acidic plant hormones (JA, ABA, SA and GAs) in plant tissues using a linear gradient profile of 0.1% formic acid in water and (A) 0.1% formic acid in methanol (B) at 0.3 mL min<sup>-1</sup>.

#### Selection of mass analyzers for plant hormone analysis

Accurate identification of the mass-to-charge ratio of the ion peak is the principle of mass spectrometry and quantification. High resolution enhances the sensitivity

of quantitative analysis [105]. One of the mainstream industries where LC–MS is constantly implemented is the doping control analysis, which employs different mass analyzers such as triple quadrupole (QqQ) [106], time of flight (TOF) [107] and quadrupole time of flight (QTOF) [108]. These qualitative and quantitative approaches are also applied in the detection of plant hormones. The use of multiple QqQ mass spectrometers to form a tandem mass spectrometry (MS/MS) not only enhances the qualitative capability of the mass spectrometer, but also retains the original quantitative capability of the quadrupole mass spectrometer. Several modes including full scan mode (Scan mode), selected ion monitor mode (SIM mode), multiple reaction monitor mode (MRM mode) and selected reaction monitor mode (SRM mode) are available in QqQ mass spectrometry [109]. Compared with the full scan mode in which all ions are simultaneously monitored, MRM mode provides higher selectivity and specificity to analyze multiple plant hormones. Fragmentation patterns of multi-class plant hormones in ESI–MS MRM mode (ET in Scan mode) are shown in Fig. 2. The conditions for MRM transition of plant hormones are listed in Table 3. Another mass analyzer widely used to detect plant hormones is TOF–MS [92]. TOF–MS exhibits faster detection speed, higher resolution and broader dynamic range of detection compared to QqQ-MS. The combination of TOF–MS and QqQ-MS provides broader application prospects (quadrupole time-of-flight mass spectrometer (QTOF-MS)) [98, 99, 110]. The quantitation of plant hormone by mass



**Table 3 Optimized MRM parameters for plant hormone detection**

Analytes	Scan mode	Precursor ion (Q1)	Collision energy (Q2)	Product ion (Q3)	References
tZ	+	220.2	26	136.1	[68]
NAA	+	185.1	− 10	158.1	[61]
6-BA	+	226.3	22	91.1	[54]
KT	+	216.0	22	148.0	[90]
IAA	−	174.0	− 14	129.6	[17]
IBA	−	203.0	− 16	158.3	[92]
IPA	−	188.0	− 16	58.9	[112]
ABA	−	263.1	− 12	153.9	[58]
JA	−	209.0	− 24	59.0	[25]
GA <sub>3</sub>	−	345.1	− 25	239.1	[113]
GA <sub>4</sub>	−	331.1	− 24	243.1	[75]
GA <sub>7</sub>	−	329.1	− 16	223.1	[63]
SA	−	137.1	− 25	92.8	[55]
BL	+	481.2	17	445.1	[20]
Sorgolactone	+	339.0	16	242	[114]

spectrometry is important for discovering the potential plant hormones in plants. Yang et al. [111] proposed identification of ripening degrees and cultivation regions of mulberries analysis, which employed high detection sensitivity and accuracy. Thus, LC–MS techniques provide an easy and robust method for plant hormone analysis.

#### Chemical labeling

Over the past decades, the trend of plant hormone analysis has lied in a tiny amount sample consumption with sensitive performance [35, 115, 116]. However, most of the plant hormones were present in plant at ultra-low levels, while many classed of them exhibit with low ionization efficiency in MS [16, 40]. Benefit from high resolution of MS, chemical labeling may serve as a strong tool to enhance sensitivity and accuracy of determining plant hormones [11, 26], which overcome the problem by synthesizing series of stable isotope-coded reagents for chemical derivatization to the target compounds during sample pretreatment or MS detection procedure [117]. The stable isotope labeling affects the accuracy of the sample pretreatment procedure and apply the possibility to directly analyze plant hormones in the extract with relative reliability [118, 119]. When combine the light and heavy isotope labeled control and sample groups, the error that introduced during sample pretreatment step would compensate and the reliable relative quantification results then obtained [120]. Li et al. [112] were simultaneous relative quantification of 8 acidic plant hormones in sub-milligram amount of plant materials by stable isotope labeling by bromocholine bromide (BETA) and its deuterated counterpart D<sub>9</sub>-BETA, which improved

sensitivity of 1–3 orders of magnitude. Furthermore, to improve the sensitivity of MS detections, amines are applied for the labelling (derivatization) procedure to form amides by making plant hormones easily ionized [31, 121]. Deng et al. [115] discovered a quaternary ammonium derivatization reagent (4-borono-*N,N,N*-trimethylbenzenaminium iodide) for ultrasensitive analysis of five BRs with vicinal diol functional groups on the side chain. The ionization efficiencies of the BRs enhanced by 1190–448,785 times and the minimal detectable amounts (MDA) of five target BRs were decreased to attomolar levels. The stable isotope labeling can improve the sensitivity and reproducibility of the plant hormone analytical procedures [19, 26, 112], which provide applicable capability in analyzing the plant hormone distribution and transportation in different plants.

#### Mass spectrometric analysis of plant hormone

##### Acidic plant hormones

##### *Auxin, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA)*

Typical acidic plant hormones comprise majority of the auxin, abscisic acid, jasmonic acid and salicylic acid [101, 102], which exhibit good response in MS negative ion mode. The quantitative analysis of these plant hormones in *A. thaliana*, wheat, *Aloe*, etc. have been realized by LC–MS/MS. Specific precursor-product ion pairs for each compound were monitored in MRM mode under the optimized fragmentor and collision energies [38, 55, 63]. A neutral CO<sub>2</sub> molecule is easily lost during the ionization of plant hormone containing carboxyl group while [M-H-CO<sub>2</sub>]<sup>−</sup> fragment ions are produced in the negative ion mode [54]. ABA only shows



good ionization efficiency in the negative ion mode and releases  $[M-H]^-$  as a molecular ion peak [17]. JA liberates  $[M-H-COOH-C_5H_9]^-$  to form MW 58.7 product ion [55].  $[M-H-R]^-$  fragment ions are produced from SA in negative ion mode [122] (Fig. 2). Specific precursor and product ions for each class of plant hormone were identified by distinctive fragmentation patterns to achieve accurately qualitative and quantitative analysis of multi-class plant hormones.

Xiao et al. [35] used a sensitive LC-MS method based on *N*-(acridin-9-yl)-2-bromoacetamide (AYBA) to examine the interactions between ABA, IAA and JA in germinated rice seeds. Good precisions with RSD 1.5–13.8% (intra-day) and 1.2–7.3% (inter-day) and acceptable recoveries (88.6–102.9%,  $n=6$ ) were achieved through this method. Luo et al. [16] developed a method for analyzing plant hormones including IAA, ABA, JA, GAs, CKs and BRs in 100 mg (FW) of *Brassica napus* L. The samples were purified and separated by sequential magnetic solid-phase extraction (MSPE) and then analyzed by an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) with the LOD ( $S/N=3$ ) ranging from 0.45 to 126.1  $\text{pg mL}^{-1}$ . The amount of plant tissues samples required has been reduced significantly from g (FW) to mg (FW). Cao et al. [70] developed a high-throughput method using liquid chromatography-triple quadrupole mass spectrometry (LC-QqQ-MS) for the profiling and quantification of 43 plant hormones and their major metabolites including auxins, ABA, JA, SA, CKs and GAs in a single sample extract. A binary solid-phase extraction with polymer anion and polymer cation exchange resins has been used as a sample pretreatment method to obtain LOD ranging from 0.03 to 29.7 fmol. The higher sensitivity of this method has enabled the detection of plant hormones at low concentration. Wu et al. [25] used in vivo SPME to detect GAs, JA and ABA plant hormones in 20 mg (FW) of aloe leaf with a spatial resolution of 3–8  $\text{mm}^3$  and obtained an LOD of 60  $\text{pg g}^{-1}$ .

### Gibberellin (GA)

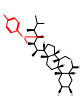
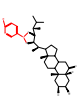
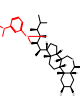
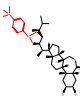
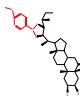
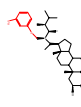
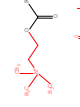
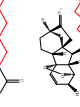

Being a class of tetracyclic diterpenoid carboxylic acids with either ent-gibberellane or 20-nor-ent-gibberellane carbon skeletons, GAs comprise of at least 136 highly similar endogenous analogs [123]. GAs appear only at ultra-trace level in plants ranging from fmol to pmol per gram (FW). Apart from their poor detectability, the complexity and the similar chemical structures of ultra-trace GAs pose a greater analytical challenge [118]. LC-MS/MS is the most widely used method for simultaneous determination of multiple GAs, however their ESI-MS analysis has poor sensitivity because of low ionization efficiency in the negative ion mode [124].

Since tetracyclic diterpenoids are stable molecules, they are easily detected by mass spectrometry as a result of fragmentation through the cleavage between functional groups. MS/MS study of GAs is usually carried out in the negative ion mode because they do not form  $[M+H]^+$  in positive ion mode. Further, in the MS/MS analysis, the secondary fragmentation of  $\text{GA}_3$  in negative ion mode  $[M-H]^-$  produces  $[M-H-CO_2]^-$  ( $m/z$  301),  $[M-H-CO_2-CO_2]^-$  ( $m/z$  257) and  $[M-H-CO_2-CO_2-H_2O]^-$  ( $m/z$  239) [125]. Under different collision energies, the high abundance of  $m/z$  143 ion is observed in the  $\text{MS}^2$  of MS/MS spectrum indicating a stable product ion of  $\text{GA}_3$  [126]. Based on the fragmentation pathway,  $m/z$  143 is generated by  $m/z$  239 ions with the loss of  $[M-H-CO_2-CO_2-H_2O-H_2O-C_6C_6]^-$  (Fig. 2). Despite the clear fragmentation pattern of GAs, the detection of GAs is still challenging due to its extremely low content [21].

Chemical-label based LC-ESI-MS/MS is commonly used for analyze GAs by introducing positively charged moieties to improve the ionization efficiency and detection sensitivity. For example, derivatization reagents with quaternary and tertiary amine group were employed for GAs labeling to enhance the ionization efficiency (the derivatization methods are mentioned in Table 4) [121, 127]. These analytical methods are beneficial for elucidating the biosynthesis and distribution of GAs in plants.

The derivatization reaction is relatively difficult since it should be carried out in mild conditions because the structure of GAs undergoes different rearrangement reactions under varying pH. For example,  $\text{GA}_3$  and  $\text{GA}_7$  undergo lactone rearrangement under alkaline conditions ( $\text{pH}>8.0$ ) while a structural rearrangement of the C/D rings and hydration of the 16,17-double bond occurs in  $\text{GA}_1$  under strong acidic conditions ( $\text{pH}<1.0$ ). Bromocholinebromide (BETA) and its deuterated counterpart  $\text{D}_9$ -BETA have been used to derivatize acidic plant hormones including GAs from sample extracts. ESI signal enhancements of 1–3 orders of magnitude were achieved under optimized conditions but a derivatization temperature of up to 95 °C was required [112]. 3-bromoacetyltrimethylammonium bromide (BTA) is used to increase the ionization efficiency of GAs. In a previous study, BTA was added prior to detection for nano-LC-ESI-Q-TOF-MS analyze GAs and LODs ranging from 1.05 to 122.4  $\text{pg mL}^{-1}$  were obtained [130]. Li et al. [19] introduced a one-reagent labeling technique by using only *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) to directly react with the ultra-trace GAs. This innovative labeling reaction was performed at pH 4.5 and 35 °C to meet the optimal conditions of pH 2.5–8.5 and a temperature of below 40 °C for analyze GAs. Protonated EDC reacts with the carboxyl group of GA to form an unstable O-acylurea, which subsequently

**Table 4 Derivatization methods and their validation parameters for plant hormone**

Analytes	Matrix	Sample pretreatment	Derivatization conditions			Derivative structure	Analytical technique	LOD	LOQ	Reference
			Reagent (v/v ratios)	°C	min					
BRs	Rice seeds (50 mg, FW)	In-tip SPE	4-PAMBA	–	–		LC-MS/MS	0.80–5.70 pg mL <sup>-1</sup> 19,000 pg mL <sup>-1</sup>	2.70– 19,000 pg mL <sup>-1</sup>	[119]
BRs	<i>A. thaliana</i> leaves (2 g, FW)	SPE	BPBA	–20 °C	2 h		UHPLC-ESI-QqQ-MS	2.00–8.00 ng L <sup>-1</sup>	6.00–23.00 ng L <sup>-1</sup>	[31]
BRs	<i>A. thaliana</i> and rice (3 g, FW)	SPE	DMAPBA	40 °C	1 h		UPLC-QTOF MS	0.51–1.17 pg g <sup>-1</sup>	1.70–3.90 pg g <sup>-1</sup>	[128]
BRs	Rice leaves (5 ± 0.1 mg FW)	PT-SPE	BTBA	80 °C	30 min		UPLC-MS/MS	0.0065– 0.021 ng g <sup>-1</sup>	0.022–0.068 ng g <sup>-1</sup>	[115]
BRs	Rice (250 mg, FW)	SPE	MPyBA	75 °C	–		UPLC-QTOF-MS	0.34–0.44 pg	1.12–1.45 pg	[129]
BRs	Tomato leaves (225 mg FW)	2D <sub>1</sub> SPE	m-APBA	–	–		HPLC-MS/MS	0.14–0.66 pg mL <sup>-1</sup>	0.47–2.20 pg mL <sup>-1</sup>	[57]
SA, IAA, IPA, IBA, ABA, JA, GA <sub>4</sub>	<i>A. thaliana</i> (0.8 mg DW)	SPE	BETA	95 °C	3 h		UPLC-MS/MS	0.61–1.85 pg mL <sup>-1</sup>	1.98–6.17 pg mL <sup>-1</sup>	[112]
SA, IAA, ABA, JA, GA <sub>5</sub>	Rice leaves (3 g, FW)	SPE	BTBA/TEA = (1/1)	105 °C	10 min		CE-ESI-TOF-MS	0.34–4.59 ng mL <sup>-1</sup>	1.12–15.3 ng mL <sup>-1</sup>	[91]
GA <sub>5</sub>	<i>A. thaliana</i> leaves (0.3 mg, FW)	MSPD	BPTAB	85 °C	–		UPLC-MS/MS	0.16–1.31 pg mL <sup>-1</sup>	0.54–4.37 pg mL <sup>-1</sup>	[118]

rearranges to a stable and amine-irreplaceable N-acylurea product through O/N migration. The sensitivity is increased by 500- to 1000-fold as compared to unlabeled GAs, furthermore, this method can directly label GAs down to about 0.1 pM as it eliminates extra reagents.

### Basic plant hormones

#### Cytokinin (CK)

At present, the number of identified CKs exceeds 40, of which nucleosides (ribosides), nucleotides, and glycosides (O and N-glycosides) are free bases. The most naturally occurring CKs are N<sub>6</sub>-substituted adenine derivatives with an isoprenoid or aromatic side chain [106, 107]. The mass spectrometry studies of CKs is usually carried out in positive ion mode, since they are easily protonated ([M+H]<sup>+</sup>) [131]. After fragmentation, the loss of nucleosyl, glycosyl, or nucleotidyl group and the fragmentation of the N<sub>6</sub>-substituent for aglycons are observed in the spectrum. The representative plant hormone for CKs is *trans*-zeatin (*tZ*) [90]. When a precursor ion is formed, the N–H bond on the *tZ* side chain cleaves to form a m/z 136 product ion (C<sub>5</sub>H<sub>4</sub>N<sub>5</sub>) [132].

Antoniadi et al. [39] applied fluorescence-activated cell sorting of green fluorescent protein (GFP)-marked cell types, combined with in-tip micro-SPE and an ultra-high-sensitive MS technique to analyze the biosynthesis and homeostasis of CK at cellular level, which revealed a LOD of 0.01–1.0 fmol. Different LC systems can influence the sensitivity of MS. For instance, a sensitive assay of cytokinins was developed using PMME/HILIC/ESI-MS/MS which enhanced the MS sensitivity for cytokinins by threefold using HILIC as compared to the use of conventional reversed phase liquid chromatography (RPLC) with a mobile phase of 85% acetonitrile with 0.01% (v/v) formic acid and 15% water with 0.01% (v/v) formic acid. The LODs for the targets ranged from 0.0028 to 0.068 ng mL<sup>-1</sup>, and the intra-day and inter-day RSD of this method were less than 12.7% [68]. The choice of liquid chromatography system mainly depends on the enrichment ability of sample pretreatment and the sensitivity of mass spectrometry. Scott et al. [133] developed a conventional HPLC system that demonstrated good sensitivity without the need for an ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) system. This method was used to screen 17 CKs (IAA, ABA and AMP), Ado and Ino within 15 min. The rapid analytical and high-throughput capabilities of this method enabled the analysis of close to 100 samples per day, with LOD ranging from 2 pM for (9G)Z to almost 750 pM for indole-3-acetic acid. This method is well suited for functional genomics platforms tailored for understanding CK metabolism.

### Neutral plant hormones

#### Brassinosteroid (BR)

All BRs contain a four-ring 5 $\alpha$ -cholestan skeleton connected to a side chain. The diversity of BR structures is caused by variations in the A and B rings as well as the substituent groups of the side chains. BRs play a crucial role in many plant developmental processes although present in trace levels (down to 0.01–0.1 ng g<sup>-1</sup> FW level) [92, 134–138]. Therefore, ideal sample pretreatment techniques should exhibit ultra-high sensitivity. Among the currently known BRs, brassinolide (BL) show the strongest activity, for BL fragmentation pattern, although the orientation of spatial position of the –OH in BL varies, ions generated from BL in the tandem mass spectrometry are substantially identical [139]. When BL fragmentation, as it shown in Fig. 2, the loss of water molecules and other neutral fragments are observed in the spectrum (BL lost two molecules of water to form the m/z 459 ion).

The commonly used detection methods such as MS are not suitable enough for BRs detection due to neutral nature with no ionizable group and the stronger hydrophobicity of BRs than other plant hormones [118, 124–126]. Therefore, the analysis of BRs requires highly selective sample preparation and highly sensitive detection with LC-MS after derivatization [29, 36, 40, 121, 126, 140]. Lv et al. [32] developed a novel hyphenated approach based on ultrasonic-assisted dispersive liquid-liquid microextraction (UA DLLME) after derivatization (using 9-phenanthreneboronic acid) for the determination of BL and obtained LOD of 8.0 ng L<sup>-1</sup>. Huo et al. [31] proposed a new labeling reagent, 2-bromopyridine-5-boronic acid (BPBA) for derivatization brassinosteroids. This is a very simple and rapid labeling procedure that remarkably increases the sensitivity of BRs detection and LOD for the three BRs of 2.0 to 8.0 ng L<sup>-1</sup>. The automated extraction of BRs can be performed by an on-line SPME with polymer monolith coupled to LC-MS for the analysis of endogenous 28-epihomobrassinolide (28-epihomoBR) in *A. thaliana*. The BR was derivatized with 3-(trimethoxysilyl) propylmethacrylate prior to detection which revealed a highly sensitive result with LOD of 2.0 ng L<sup>-1</sup> [34]. The combination of several purification methods provides an efficient and sensitive processing of plant samples. Recently, Wang et al. [37] quantified six endogenous BRs with good compatibility and sensitivity by eliminating non-polar, polar and ionizable interferences using a matrix in-line MSPD-MAX-MCX coupled with HPLC-MS/MS to obtain LODs ranging from 0.008 to 0.04 ng mL<sup>-1</sup>. In another study, in situ derivatization (ISD) was introduced and coupled with multiple sample preparation methods to simplify the process of extraction and derivatization [141]. Luo et al. [42] developed a solid phase boronate affinity labeling (SPBAL) and extraction

technique, followed by a desorption & salt-induced phase transition extraction (SPTE) for further purification to rapidly determine endogenous BRs in plant tissues. The addition of a boronate affinity labeling reagent (4-PAMBA) for ISD lead to a 923–15,000-fold increase in sensitivity of BRs detection, with LODs ranging between 1.4 and 2.8 pg mL<sup>-1</sup>.

### **Strigolactone (SL)**

Strigolactones (SLs) have been suggested to act as a long-distance branching factor that suppresses the growth of preformed axillary shoot buds. They are derived from carotenoids and were recently recognized as a new family of plant hormone [59, 91, 111, 142]. Typically, the natural SLs identified so far consist of tetracyclic skeleton (A, B, C, and D rings) with a tricyclic lactone (ABC rings) connected by an enol ether group to an  $\alpha,\beta$ -unsaturated furanone moiety (D ring) [114]. SLs show relatively lower ionization efficiency because of their neutral structure which is similar to BRs which cause interference during MS detection. The lactone structure of C and D rings in SL molecules exhibits lower stability as they can hydrolyze in acidic or alkaline conditions leading to a more complicated sample preparation and instrumental analysis [44]. Except for the same structure of D ring, some of the ABC rings in SLs contain –OH, –CH<sub>3</sub>, –COOH, –CH<sub>2</sub>CO and –O– groups. The compounds with these functional groups including D ring can easily be cleaved to form fragment ions during the cleavage process in MS detection. SLs are usually detected in the positive ion mode. A typical SLs-GR24 (C<sub>17</sub>H<sub>14</sub>O<sub>5</sub>) has a molecular weight of 298, and the main fragment ions formed are m/z=97 and m/z=185 [44, 114, 143, 144]. The typical product ion of SLs (m/z=97) is mainly the enol ether found between the ABC ring and the D ring in the GR<sub>24</sub> structure [144].

Yoneyama et al. [114] analyzed SLs in root exudates from 12 *Fabaceae* plants employing LC–MS/MS to clarify the regulation of SL production and revealed that exudation is closely related to the nutrient acquisition strategy of plants. Xie et al. [44] also used LC–MS/MS to characterize five different stimulants including four SLs from the root exudates of tobacco providing a valuable analytical strategy for determination of SLs. Foo et al. [143] used UPLC-MS/MS to quantify SL levels in the root exudate of 30-day-old rms1-2T and wild-type plants using deuterium-labeled internal standards. This was the first direct evidence that shooting does not majorly contribute to the SL levels in roots. Kohlen et al. [144] performed SL detection using HPLC -MS/MS, and the MRM transitions of [M+H/Na]<sup>+</sup>>[M+H/Na-D ring]<sup>+</sup> and [M+H/Na]<sup>+</sup>>[D ring]<sup>+</sup> were selected. Two SLs (orobanchol and orobanchyl acetate) were identified in

*Arabidopsis* and the presence of a third (5-deoxystrigol) SL was discovered. These results show that xylem-transported SLs contribute to the regulation of shoot architectural response to phosphate-limiting conditions.

### **Conclusions**

Major technological advancements in sample pretreatment methods and mass spectrometric methods in recent years has substantially facilitated plant hormone analysis [117, 145–148]. To date, MS studies have identified many plant hormones and their metabolites that might be involved in the molecular mechanisms and physiological functions of plant development, but the analysis at unicellular level has remained a challenge. Several large-scale studies on plant hormone have been reported, these studies have promoted our understanding on the dynamic spatial–temporal distribution of plant hormone [19, 35, 77]. Current sample pretreatment methods for plant hormone is expected to be further optimized for realizing the in situ, real-time and high spatial resolution. The frontier research of analytical methodology will broaden the development of sample pretreatment techniques and derivatization strategies for more accurate characterization of plant hormone.

Going forward, the existing methods need to be improved to enhance the efficiency of sample treatment and chromatographic-mass spectrometry analysis of plant hormone in the following aspects:

1. Sample pretreatment methods with the ability to achieve real-time in situ and in vivo analysis of the spatial–temporal distribution of plant hormone are required to facilitate further studies on plant hormone regarding the synthesis, metabolism, transportation pathway and functional effects. Therefore, it is anticipated that the development of highly efficient sample pretreatment methods for in vivo and real-time analysis. The efficiency methods are likely to dominate future research to improve the characterization of the distribution of multi-class plant hormones within a whole plant or specific plant organs in single cell orientation.
2. Several methods have been developed for multi-class plant hormones analysis, but even the most influential methods are expected to improve the selectivity prior to analyzing different plant hormones isomers belonging to the same class with structural similarities. Recent advancements in the analytical methods with micro-separation ability, excellent selectivity and high sensitivity features would facilitate more comprehensive real-time monitoring of plant hormone during growth and development.

3. Mass spectrometric analysis is highly suitable for plant hormone analysis and has witnessed widespread application in recent years. Further studies would anticipate to develop derivatization methods for ultra-trace compounds analysis, as derivatization methods improve the analytical sensitivity and the spatial–temporal resolution of plant hormone. Furthermore, the establishment of a mass spectral database for plant hormone is crucial for accurate qualitative and quantitative analysis. Lastly, such a database may act as a tool for advancing scientific theories and for the potential discovery of new plant hormones.

#### Abbreviations

CK: Cytokinin; ABA: Abscisic acid; GA: Gibberellin; BR: Brassinosteroid; SA: Salicylic acid; JA: Jasmonic acid; ET: Ethylene; SL: Strigolactone; FW: Fresh weight; MS: Mass spectrometry; GC-MS: Gas chromatography–mass spectrometry; LC-MS: Liquid chromatography–mass spectrometry; IT: Ion trap; Q-TOF: Quadrupole time-of-flight; QQQ: Triple quadrupole instruments; LLE: Liquid–liquid extraction; LPME: Liquid phase microextraction; DLLME: Dispersive liquid–liquid microextraction; PMME: Polymer monolith microextraction; SPE: Solid phase extraction; SPME: Solid phase microextraction; MSPE: Magnetic solid phase extraction; SBSE: Ion pair stir bar sorptive extraction; EME: Electromembrane extraction; CE: Capillary electrophoresis; DSPE: Dispersive solid-phase extraction; HPLC: High performance liquid chromatography; UHPLC: Ultra high performance liquid chromatography–mass spectrometry; RPLC: Reversed phase liquid chromatography; LOD: Limit of detection; LOQ: Limit of quantitation; –CD: Cyclodextrin; NAA: Naphthaleneacetic acid; 2-NOA: 2-Naphthoxyacetic acid; tZ: Trans-zeatin; 28-epihomoBR: 28-Epihomobrassinolide; poly(MAA-co-EDMA): Poly(methacrylic acid-co-ethylene dimethacrylate); RSD: Relative standard deviation; ESI: Electrospray ionization; APCI: Atmospheric pressure chemical ionization; ACN: Acetonitrile; QqQ: Triple quadrupole; TOF: Time of flight; QTOF: Quadrupole time of flight; MS/MS: Tandem mass spectrometry; Scan mode: Scan mode; SIM mode: Selected ion monitor mode; MRM mode: Multiple reaction monitor mode; SRM mode: Selected reaction monitor mode; ESI-MS: Electrospray ionization mass spectrometry; QTOF-MS: Quadrupole time-of-flight mass spectrometer; LC-QqQ-MS: Liquid chromatography–triple quadrupole mass spectrometry; –COOH: Carboxyl groups; –OH: Hydroxyl groups; AYBA: *N*-(acridin-9-yl)-2-bromoacetamide; BETA: Bromocholinebromide; BTA: 3-Bromoactonyltrimethylammonium bromide; EDC: *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride; SPBAL: Solid phase boronate affinity labeling; SPTE: Salt-induced phase transition extraction.

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#### Authors' contributions

LW and HYK analyzed references and drafted the manuscript; YZ, GW HS, and LC assisted to collection and analysis of references, LM and DL proposed the subject and revised manuscript, CL provided financial supports. All authors read and approved the final manuscript.

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#### Consent for publication

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#### Competing interests

The authors declare that they have no competing interests.

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