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REVIEW

Quo vadis plant hormone analysis?

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Abstract Plant hormones act as chemical messengers in the regulation of myriads of physiological processes that occur in plants. To date, nine groups of plant hormones have been identified and more will probably be discovered. Furthermore, members of each group may participate in the regulation of physiological responses *in planta* both alone and in concert with members of either the same group or other groups. The ideal way to study biochemical processes involving these signalling molecules is 'hormone profiling', i.e. quantification of not only the hormones themselves, but also their biosynthetic precursors and metabolites in plant tissues. However, this is highly challenging since trace amounts of all of these substances are present in highly complex plant matrices. Here, we review advances, current trends and future perspectives in the analysis of all currently known plant hormones and the associated problems of extracting them from plant tissues and separating them from the numerous potentially interfering compounds.

Keywords Plant hormones · Extraction · Isolation · Mass spectrometry · Liquid chromatography · Gas chromatography

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Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
BRs	Brassinosteroids
CE	Capillary electrophoresis
CKs	Cytokinins
GAs	Gibberellins
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
JA	Jasmonic acid
JAs	Jasmonates
LC	Liquid chromatography
MS	Mass spectrometry
SA	Salicylic acid
SLs	Strigolactones
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry

Introduction

Most (if not all) organisms use chemical signals in cell–cell communication. Thus, chemical signalling is extremely ancient. However, the complexity of cell signalling leapt when first prokaryotic and subsequently eukaryotic cells began to associate together in multicellular organisms, putatively several billion and one billion years ago, respectively (Parfrey et al. 2011). Following the emergence of multicellularity, cell specialisation increased as tissues and organs with diverse specific functions evolved. Co-ordination of the growth and development of these cells,

tissues and organs, as well as the environmental responses of complex multicellular organisms, required increasingly intricate signalling networks. Many of our current concepts about intercellular communication in plants have been derived from similar studies in animals, in which two main systems evolved: the nervous system and endocrine system. Plants, lacking motility, never developed a nervous system, but they did evolve hormones as chemical messengers. Plant hormones play essential roles (individually and in concert) in the regulation of myriads of physiological processes involved in plants' growth, development, senescence and responses to environmental stimuli. Until the 1990s, there were just five known types of plant hormone: auxins, cytokinins, gibberellins, ethylene and abscisic acid. However, during the last two decades compelling evidence has emerged that four other classes of substances (brassinosteroids, jasmonates, salicylic acid and most recently strigolactones) act as signalling molecules and probably have growth-regulating activities.

Plant hormones, also known as 'phytohormones', are usually present at extremely low concentrations in plant tissues, generally pg/g fresh weight (FW), while substances that interfere with their analysis are present in far greater concentrations. This is the major problem associated with plant hormone analysis. Thus, sound knowledge of the analytical and chemical principles underlying the extraction, purification, identification and quantification of plant hormones is essential for their accurate and precise determination. In this review, we summarise current understanding of these principles, methodologies for plant hormone analysis, factors that complicate their extraction and isolation from the highly complex matrices of plant and other tissues (which contain thousands of substances) and future perspectives.

Extraction and purification

Prior to extraction, plant material must be homogenised to break the cell walls in the tissues (Harrison 2011) and thus allow any hormones present to migrate to an appropriate extraction solvent. This can be done by grinding freeze-dried or fresh plant tissue (gram amounts) in a mortar with a pestle under liquid nitrogen then adding an appropriate solvent to the ground material. Alternatively, very small amounts of plant material (mg) can be ground in 1.5–2.0 ml plastic microtubes with a selected extraction solvent and tungsten carbide or zirconium oxide beads in a homogenizer for an appropriate time at a selected frequency. The most effective devices use multi-directional motions to transmit high kinetic energy to the beads and are capable of grinding tens of samples simultaneously. To avoid enzymatic or chemical degradation of the hormones, the plant material should be kept cold during the entire homogenisation process. The efficiency of extraction of a target hormone from a plant tissue will depend on its polarity, its subcellular localisation and the extent to which it is associated with other compounds in the tissue such as phenolics, lipids, pigments and proteins (Hillman 1978). The solvent used must be capable of extracting the hormone efficiently, while minimising extraction of interfering substances. Methanol, acetonitrile, mixtures of these solvents with aqueous solutions of organic acids (generally formic or acetic) or buffers adjusted to neutral pH are usually used as extraction solvents for isolating plant hormones (Kowalczyk and Sandberg 2001; Nordström et al. 2004; Novák et al. 2008; Kojima et al. 2009; Urbanová et al. 2013). Analyte losses during the sample purification procedure can be accounted for by adding internal standards (usually labelled with stable isotopes) to the plant extracts. This procedure also provides a measure of the percentage recovery of target metabolites throughout the purification procedure. Ideally, recovery markers should be included for every plant hormone metabolite that is being quantified. However, in many studies only a few internal standards have been used, often added at late stages during the extraction process, or even just before quantitative analyses (Witters et al. 1999; van Rhijn et al. 2001). Clearly, all the current methodologies could be further improved by sophisticated internal standardisation of some of the missing labelled standards, mainly to cover the enormous variations in chemical properties of the substances, even within each phytohormone group. Dissimilar chemical nature of endogenous and internal substances subsequently leads to errors in their determination.

The ideal extraction duration depends on the target plant hormone group and (to a lesser degree) the specific target hormones. Generally, it should be long enough to allow quantitative migration of the analytes into the extraction medium and isotopic equilibration between the endogenous compounds and added internal standards. Decomposition of the endogenous hormones during prolonged extractions can be minimised by performing the extraction at low temperature (between −20 and 4 °C) and adding an appropriate antioxidant (for instance diethyldithiocarbamic acid; Pěnčík et al. 2009) to the extraction solvent.

The optimal purification method depends on the chemical nature of the target hormones, the type of analysis to be performed and choice of analytical instrument. In addition, appropriate separation procedures must be applied to reduce levels of interfering compounds in the extracts while maximising recoveries of the hormones in each purification step (Ljung et al. 2004). The first step is often liquid–liquid extraction combined with solid-phase extraction (SPE). SPE columns are packed by the manufacturers with solid sorbents that bind plant hormones (and other compounds, to varying degrees), usually via hydrophobic, polar or ionic interactions (often sorbents with hydrocarbon groups, graphitized carbon-based material and ion-exchange matrices,

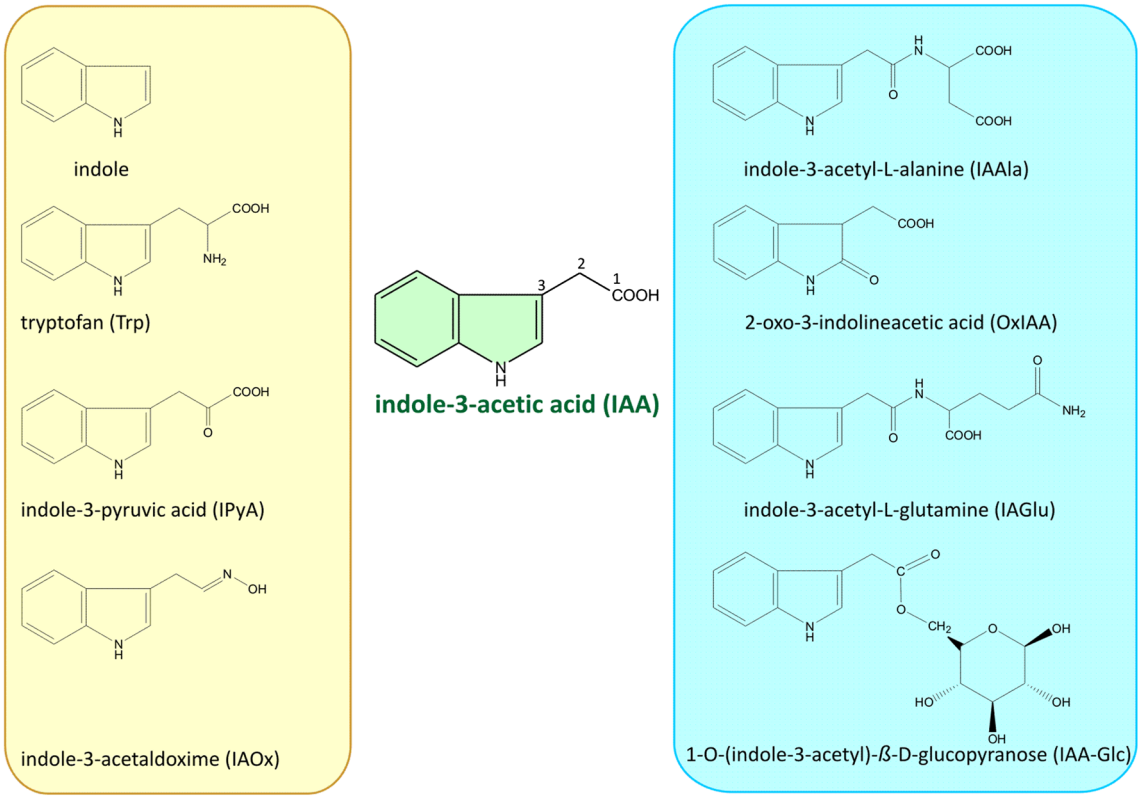


Fig. 1 Structures of auxins

respectively). Interfering substances are removed by washing the column with a suitable solvent and hormones are then eluted using a solvent that disrupts the bonds formed by the interactions between the hormones and the sorbent in the column. "Mixed-mode" SPE columns, packed with a mixture of two types of sorbent, are also available and have become very popular recently (Nordström et al. 2004; Dobrev et al. 2005; Novák et al. 2008; Kojima et al. 2009; Urbanová et al. 2013) due to their ability to reduce the number of required purification steps (since more than one separation mechanism can be exploited using a single column), while maintaining high sample clean-up efficiency. SPE allows high throughput of samples when combined with automatic systems, SPE robots, which are capable of purifying tens of sample simultaneously (Nordström et al. 2004; Kojima et al. 2009). However, no miniature mixed-mode purification system capable of handling extracts from mg FW samples has been developed yet.

Auxins

Auxins were the first discovered family of plant hormones. In the earliest recorded inference, Charles and Francis Darwin concluded that plant growth is regulated by a signal transported from one part of the plant to another where the physiological growth response occurs (Darwin and Darwin 1880). This "signal" was subsequently called auxin (from the Greek word "auxein" meaning "to grow") and identified as indole-3-acetic acid (IAA; Kögl and Kostermans 1934; Went and Thimann 1937). IAA (Fig. 1) is the major auxin involved in a plethora of physiological processes in plants. Its activities include induction of cell division and elongation in stems, and regulation of cell differentiation, various tropisms, abscission, apical dominance, senescence and flowering (Woodward and Bartel 2005; Teale et al. 2006). Two major IAA biosynthesis pathways have been postulated in plants: the tryptophan (Trp)-independent and Trp-dependent pathway (Normanly 2010; Mano and Nemoto 2012). After synthesis, IAA may be deactivated by catabolic oxidation (decarboxylative or non-decarboxylative), or conjugation to sugars and amino acids (Normanly 2010; Ljung 2012).

To obtain complete understanding of IAA metabolism in a given biological sample, information on levels of free hormone, its major metabolites and biosynthetic precursors is highly important. Accurate estimation of these substances requires the detection and quantification of minute amounts of analytes in plant extracts containing huge numbers of other substances at far higher concentrations.

Therefore, it is essential to use methodology that offers low detection limits and high selectivity, i.e. methods that are minimally sensitive to impurities. Several methods have been described for detecting free IAA, including HPLC with fluorescence detection (Crozier et al. 1980; Sundberg et al. 1986; Mattivi et al. 1999; Dobrev et al. 2005) or chemiluminescence detection (Xi et al. 2009), with or without enhancement by immunoaffinity-based purification techniques (Pengelly et al. 1981; Sandberg et al. 1985; Cohen et al. 1987; Marcussen et al. 1989). However, the most commonly employed method for quantifying IAA in plant tissues seems to be gas chromatography–mass spectrometry (GC–MS) with electron impact ionisation (Chen et al. 1988; Dunlap and Guinn 1989; Edlund et al. 1995; Ribnicky et al. 1998; Perrine et al. 2004; Barkawi et al. 2010). A drawback of this approach is that IAA is not volatile so it must be derivatised (usually by methylation or trimethylsilylation). Several methods for preparing derivatives of IAA precursors for GC–MS analysis have also been developed, including acylation of tryptamine, trimethylsilylation of indole-3-ethanol, and methyl chloroformate derivatisation of tryptophan (Quittenden et al. 2009; Liu et al. 2012a, b, c). Samples can be purified by reversed-phase SPE (Barkawi et al. 2008), mixed-mode SPE (Dobrev et al. 2005) or immunoaffinity extraction (Sundberg et al. 1986; Pěnčík et al. 2009). To avoid preparation of antibodies in animals, selective binding in a polymer matrix with a "molecular imprint" (MIP) of auxin can be used (Zhang et al. 2010). A miniature system for purifying IAA and its biosynthetic precursors using SPE tips has been developed (Liu et al. 2012a, b, c), and the best currently available analytical technology is based on liquid chromatography-tandem mass spectrometry (LC–MS/MS), which is capable of determining both IAA and its amino acid conjugates (Kowalczyk and Sandberg 2001; Pěnčík et al. 2009). However, this requires a much more intricate procedure than measurements of IAA alone, mainly because levels of IAA conjugates in plant extracts are significantly lower. However, all IAA metabolites except indole-3-pyruvic acid (IPyA, Fig. 1) can be analysed without any derivatisation prior to their MS detection in positive or negative electrospray mode (Kai et al. 2007a, b; Sugawara et al. 2009; Mashiguchi et al. 2011; Novák et al. 2012). Recently, IPyA (the most labile auxin precursor) has been identified as an important intermediate in the Trp-dependent IAA biosynthesis pathway in Arabidopsis (Mashiguchi et al. 2011; Stepanova et al. 2011). Tam and Normanly (1998) described a simple, rapid method for its reliable quantification based on derivatisation of the carbonyl group by hydroxylamine to form the oxime. Other methods, such as derivatisation of IPyA by 2,4-dinitrophenylhydrazone (Mashiguchi et al. 2011), cysteamine (Novák et al. 2012) or sodium borodeuteride (Liu et al. 2012a, b, c) have also been developed.

Cytokinins

Cytokinins (CKs) are endogenous N^6-substituted adenine derivatives with the well-known primary ability to induce cell division activity in plant callus cultures (Skoog and Miller 1957). However, they also have a very wide spectrum of other physiological effects on various plants and tissues, notably they can delay senescence, inhibit root growth and branching, increase resistance to environmental stresses and initiate seed development (Richmond and Lang 1957; Mok 1994). As shown in Fig. 2, CKs can be divided into two subgroups based on their chemical structure: isoprenoid CKs (ISCKs), which bear an isoprenoid side chain at position N-6 and include zeatin, isopentenyl and dihydrozeatin forms; and aromatic CKs (ARCKs), which bear a side chain of aromatic (benzyl or furfuryl) origin. From a physiological perspective, there are four main types of CK metabolism: interconversion, hydroxylation, conjugation and oxidative degradation. However, the

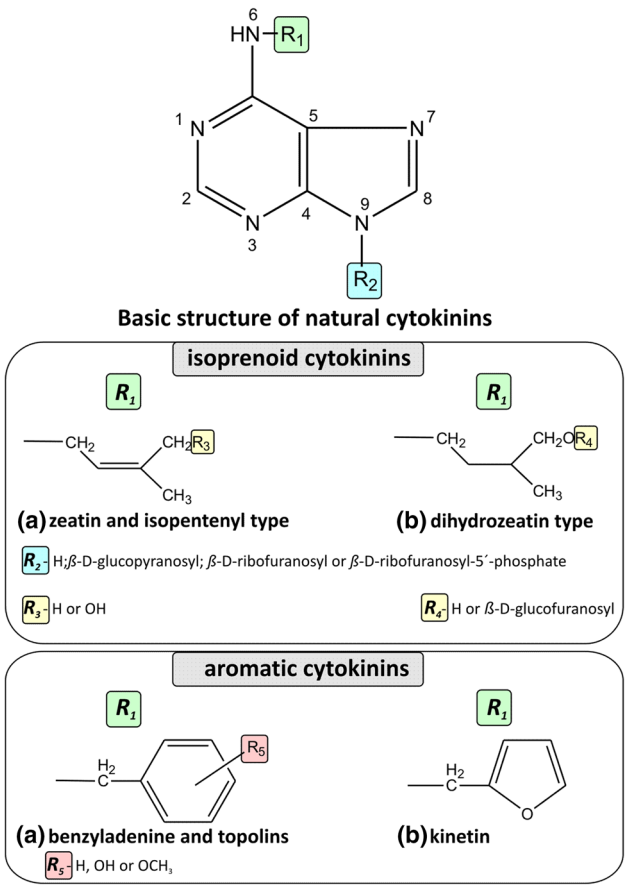


Fig. 2 Structures of cytokinins

major CK metabolic processes are interconversions of CK bases, nucleosides and nucleotides (Chen 1981), as rates of CK nucleoside and nucleotide conversions to bases (the biologically active forms) reportedly control CK activity in plant cells (Kurakawa et al. 2007). Side chain modifications of ISCKs include stereospecific hydroxylation of the isopentenyl side chain, yielding zeatin (Takei et al. 2004), and reduction of the zeatin side chain, yielding dihydrozeatin (Mok and Martin 1994). Zeatin occurs naturally as two geometric isomers: *trans*- and *cis*-zeatin. In general, *trans*-zeatin (*t*Z) is considered as a cytokinin with high activity, compared to the little or no active *cis*-zeatin (*c*Z) (Kudo et al. 2012). Early investigators postulated that *t*Z was the predominant form, while the *cis*-isomer was much less abundant *in planta* (Schmitz and Skoog 1972; Mok et al. 1978). However, there are growing indications that *c*Z is the dominant cytokinin species in various plants, such as rice (Takagi et al. 1985), maize (Veach et al. 2003; Vyroubalová et al. 2009), potatoes (Suttle and Banowetz 2000) and several species of legumes (Emery et al. 1998, 2000; Quesnelle and Emery 2007). Interestingly, relative levels of zeatin stereoisomers can also differ substantially during a plant's lifecycle, *c*Z-type CKs generally predominate in tissues exposed to various stresses (drought, heat or biotic stress), while *t*Z-type CKs are often more abundant in unstressed tissues (Havlová et al. 2008; Pertry et al. 2009; Vyroubalová et al. 2009; Dobra et al. 2010). Common modifications of ARCK side chains are regiospecific hydroxylations, leading to formation of either *meta*- or *ortho*-derivatives called topolins (Kamínek et al. 1987; Strnad 1997). *Meta*-position of hydroxyl functional group increases CK activity of the parent compound, while at the *ortho*-position leads to its decrease (Holub et al. 1998). Glycosylation, leading to the formation of *N*- or *O*-glycosides of CKs, also occurs in many plant species (Entsch et al. 1979). *N*-glycosides lack CK activity in bioassays, indicating that their formation is a form of irreversible inactivation (Laloue 1977). In contrast, *O*-glycosides are considered inactive storage forms that play important roles in balancing CK levels (McGaw and Burch 1995). Free CK bases and nucleosides with unsaturated N^6-side chains may be irreversibly degraded by cleavage of the side chain catalysed by cytokinin oxidase/dehydrogenase, yielding adenine or adenosine and the corresponding side chain aldehyde (Galuszka et al. 2001).

CK metabolites have significantly differing chemical properties that must be considered in analyses. Notably, their ionic forms are dependent on pH, which thus strongly influences their behaviour on ion-exchange columns. For instance, at a pH of ca. 2, CK nucleotides are zwitterionic (uncharged), while CK bases and several metabolites (including 9-ribosyl and 3-, 7- and 9-glycosyl metabolites) are cationic. In addition, nucleotides are more polar and thus less hydrophobic than glycosides, which in turn are more polar and less hydrophobic than CK bases and ribosides. Thus, CK metabolites' chromatographic properties vary widely, which complicates their analysis. In the 1960s, during the GC boom, both GC–MS and GC–ECD techniques were introduced for CK analysis. However, chemical modification of hydrogen-binding functional groups was essential for converting CKs (which are not volatile; Horgan and Scott 1987) into volatile derivatives suitable for GC. Various derivatisation approaches have been published, including trimethylsilylation (TMS, Most et al. 1968), permethylation (Morris 1977) and trifluoroacetylation (TFA, Ludewig et al. 1982). However, these procedures are associated with a number of technical difficulties, such as requirements for extremely water-sensitive reagents, inappropriate and time-consuming preparation, the extreme sensitivity of some derivatives (TMS and TFA) to moisture, and the need for high temperatures to elute permethylated derivatives. To avoid the problems arising from CK derivatisation for GC, attention has focused on LC–MS. The first LC–MS method for CK analysis, involving the separation of underivatised cytokinins using a frit-fast atom bombardment interface, was published by Imbault et al. (1993). The sensitivity of this method was subsequently improved, to low femtomolar detection limits, by derivatising 10 ISCKs using propionyl anhydride to form CK propionyl derivatives (Åstot et al. 1998; Nordström et al. 2004). In addition, atmospheric pressure ionisation (APCI, Yang et al. 1993) and electrospray ionisation (ESI, Prinsen et al. 1995; Witters et al. 1999; Novák et al. 2003) interfaces have been used for CK determination, affording picomolar to low femtomolar detection limits in analyses of 0.1–1 g FW samples of plant tissue. Nowadays, ESI is the only MS interface routinely used for quantitative analysis of CKs that offers sufficient ionisation efficiency not only for CK but also for the majority of plant hormones (Novák et al. 2008; Svačinová et al. 2012; Farrow and Emery 2012; Dewitte et al. 1999). Since CKs strongly absorb UV light (in the 220–300 nm region), several LC–UV methods have been earlier applied for quantitative analysis of CKs (Campell and Town 1991; Chory et al. 1994). UV detection can be further advantageous for analyses of immunoaffinity-purified cytokinin samples (Nicander et al. 1993) and separation of CKs by capillary electrophoresis (CE; Pacáková et al. 1997; Béres et al. 2012). In some cases, CE has been found to have distinct advantages over ultra-high performance liquid chromatography (UHPLC) in terms of separation efficiency, costs and simplicity, while maintaining comparable sensitivity to MS detection (Ge et al. 2006).

As substituted purine derivatives CKs also have typical electroactive properties, so they can be detected by electrochemical reduction or oxidation using appropriate electrodes (Hernández et al. 1995; Hušková et al. 2000;

Tarkowská et al. 2003). However, these methods are more useful for screening purposes than routine analysis of endogenous cytokinin levels in plant tissues.

Similarly to other plant hormones, numerous attempts have been made to increase the sensitivity, peak capacity and speed of analyses of the trace quantities of CKs present in small amounts of various plant tissues (e.g. apical roots, stem regions, seeds and buds) or even individual cell organelles. Such requirements can be fulfilled by UHPLC in combination with tandem MS. However, extremely careful attention must also be paid to the efficiency of CK extraction and isolation from plant matrices, which (as mentioned) are very complex and typically contain thousands of substances. SPE followed by a high-throughput batch immunoextraction step and subsequent UHPLC separation has proved to be highly valuable for this, allowing for example the separation of 50 CKs—including bases, ribosides, 9-glycosides, *O*-glycosides and nucleotides—from several milligrams of poplar (*Populus* × *canadensis* Moench, cv *Robusta*) leaves (Novák et al. 2008).

Recently, miniature sample pretreatments based on hydrophilic interaction liquid chromatography (HILIC) combined with MS/MS have also been used for CK analysis (Liu et al. 2010, 2012a). Further improvements allowing reductions of starting amounts of tissue while maintaining sensitivity have been achieved by miniaturisation of SPE apparatus from polypropylene columns to pipette tips, so-called stop-and-go-microextraction or StageTip purification, which affords attomolar detection limits using 1–5 mg FW of *Arabidopsis* seedlings (Svačinová et al. 2012). In addition, magnetic solid-phase extraction techniques, involving use of magnetic or magnetizable adsorbents with high adsorption ability and superparamagnetism, have been introduced for effective sample enrichment and purification of CKs prior to HILIC combined with tandem mass spectrometry (Liu et al. 2012b). This approach was applied to analyse CKs in 200 mg FW extracts of rice roots (*Oryza sativa*) and *Arabidopsis thaliana* seedlings with pg/mL detection limits. Another approach for improving sample enrichment is to selectively bind CKs from plant extracts using molecularly imprinted polymers (MIPs) prior to LC–MS/MS analysis. This method was developed and applied to estimate levels of two 2 ISCKs and two ARCKs in 5 g FW extracts of tobacco, rape and soybean leaves with pg/mL detection limits (Du et al. 2012).

Gibberellins

Gibberellins (GAs) are a class of diterpenoid carboxylic acids that include biologically active compounds produced by various microbes (fungal and bacterial) and lower as well as higher plants, where they are endogenous growth regulators. To date, 136 naturally occurring GAs from diverse natural sources have been characterised (http://www.plant-hormones.info/gibberellins.htm). The most prominent physiological effects of bioactive GAs (e.g. GA_1, GA_4, Fig. 3) include for instance induction of flowering and germination, stimulation of stem elongation and delay of senescence in leaves and citrus fruits (Hedden and Thomas 2012).

All naturally occurring GAs possess a tetracyclic *ent*-gibberellane skeleton consisting of 20 carbon atoms (with rings designated A, B, C and D; Fig. 3), or a 20-nor-*ent*-gibberellane skeleton (in which carbon-20 is missing, so there are only 19 carbon atoms). Therefore, in terms of carbon numbers, GAs can be divided into two groups: C_{19}-GAs (e.g. GA_4, GA_1) and C_{20}-GAs (e.g. GA_{12}, GA_{53}). The prefix *ent* indicates that the skeleton is derived from *ent*-kaurene, a tetracyclic hydrocarbon that is enantiomeric to the naturally occurring compound kaurene. Like other classes of plant hormones, concentrations of GAs in plant tissues are usually extremely low (generally pg/g FW). Thus, very sensitive analytical methods are required for their detection. However, levels of GAs may vary substantially even within a plant organ. Vegetative tissues (stems, roots and leaves) typically contain several pg/g FW, while reproductive organs (such as seeds and flowers) often have three orders of magnitude higher levels (i.e. ng/g FW). The chemical nature of GAs also varies substantially, notably

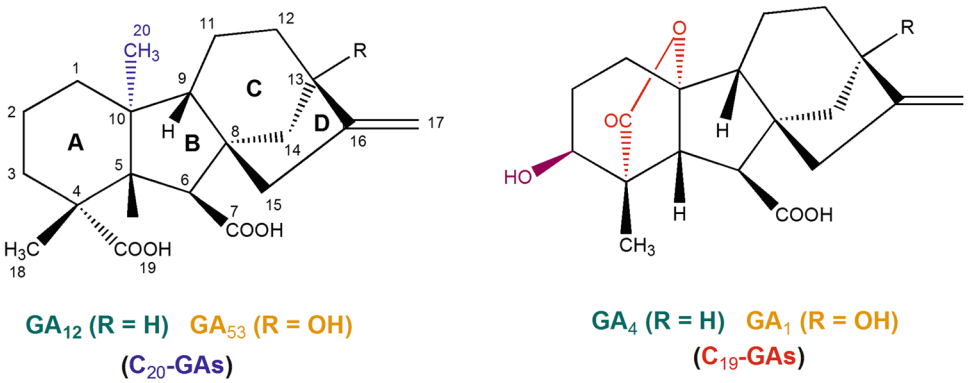


Fig. 3 Structures of C_{19} and C_{20} gibberellins

they cover a broad range of polarities and the only properties they share are that they behave as weak organic acids, with dissociation constants (pK_a) around 4.0 (Tidd 1964), and have no spectral characteristics such as fluorescence or UV absorption (except below 220 nm) that could easily distinguish them from other organic acids. The first methods for GA analysis, based on GC–MS determinations of volatile methyl ester trimethylsilyl ether derivatives, were introduced in the 1960s (Pryce et al. 1967; MacMillan and Pryce 1968; Binks et al. 1969). This approach is still used in some laboratories for quantifying and identifying GAs as it is highly sensitive (Mauriat and Moritz 2009; Magome et al. 2013). However, LC–MS is becoming more popular for quantitative analysis of GAs, mainly because it avoids derivatisation requirements. For instance, Varbanova et al. (2007) published a method for analysing 14 GAs in extracts of *Arabidopsis* mutants within 16 min by LC–MS/MS (after a laborious five-step purification procedure). The quantification procedure involved addition of deuterium-labelled internal standards before purification followed by isotope dilution analysis, as generally recommended for precise quantification (Croker et al. 1994). LC–MS/MS-based analysis has also been successfully used to determine endogenous GAs in Christmas rose (*Helleborus niger* L.) during flowering and fruit development (Ayele et al. 2010). Most recently, a rapid, sensitive method based on a two-step isolation procedure followed by UHPLC-MS/MS analysis has been published (Urbanová et al. 2013). This methodology is capable of quantifying 20 naturally occurring biosynthetic precursors, bioactive GAs and metabolic products from extracts of 100 mg FW plant tissues with low femtomolar detection limits.

Abscisic acid

Abscisic acid (ABA) is an optically active C_{15} terpenoid carboxylic acid (Fig. 4) that was discovered during the early 1960s, when it was found to be involved in the control of seed dormancy and organ abscission (Liu and Carns 1961; Ohkuma et al. 1963; Cornforth et al. 1965). Later, it was shown that the role of ABA in regulating abscission is minor and its primary role is in regulating seed dormancy and stomata opening (Patterson 2001). ABA plays important roles in many other numerous physiological processes such as seed maturation, adaptive responses to abiotic stress (Nambara and Marion-Poll 2005), shoot elongation, morphogenesis of submerged plants (Hoffmann-Benning and Kende 1992; Kuwabara et al. 2003), and root growth maintenance (Sharp and LeNoble 2002). It is a non-volatile, relatively hydrophobic substance containing a carboxylic group (Fig. 4). Therefore, commonly applied approaches for its extraction and purification include liquid–liquid extraction (Liu et al. 2002; Schmelz et al. 2003; Durgbanshi et al. 2005), liquid–liquid–liquid microextraction (Wu and Hu 2009; Bai et al. 2012), SPE (Dobrev and Kamínek 2002; Chiwocha et al. 2003; Zhou et al. 2003; Dobrev et al. 2005) and solid-phase microextraction (Liu et al. 2007). Like other phytohormones, it was initially determined by bioassays based on its physiological properties (Sembdner et al. 1988). The naturally occurring form is S-(+)-ABA, and the side chain of ABA is in 2-*cis*, 4-*trans* configuration by definition (Addicott et al. 1968). Due to this optical property, ABA was also previously determined by polarimetry (Cornforth et al. 1966). However, specific rotation is often influenced by numerous other substances

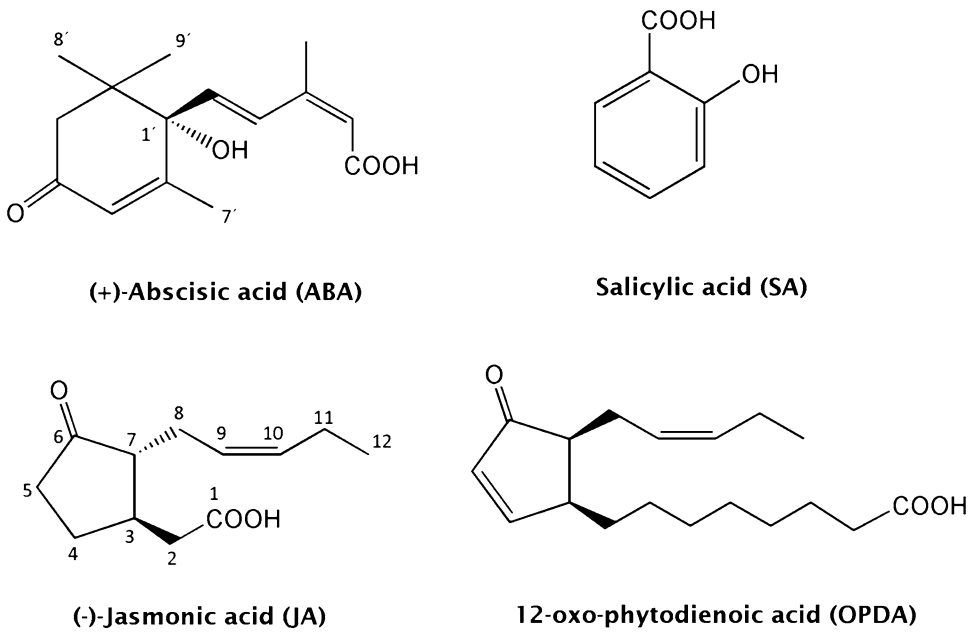


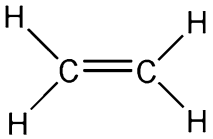
Fig. 4 Structures of stress-related plant hormones

in plant extracts, thus such determination is very inaccurate. The compound also strongly absorbs ultraviolet (UV) radiation, maximally at about 260 nm (due to the presence of chromophores, chemical groups capable of absorbing light, resulting in the colouration of organic compounds), which allows its detection in HPLC eluates by monitoring their UV absorption (Ciha et al. 1977; Cargile et al. 1979; Mapelli and Rocchi 1983). HPLC has also been used to determine two metabolites of ABA: phaseic acid (PA) and dihydrophaseic acid (DPA) (Durley et al. 1982; Hirai and Koshimizu 1983).

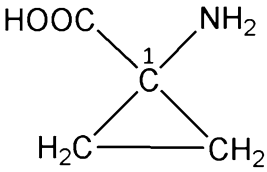
Immunological methods such as radioimmunoassays (RIAs) (Weiler 1979, 1980; Walton et al. 1979; Mertens et al. 1983) and enzyme-linked immunosorbent assays (ELISAs) based on competitive binding between free and alkaline phosphatase-labelled ABA (Daie and Wyse 1982; Weiler 1982) have also been successfully used for estimating ABA levels and are still highly recommended for estimating free ABA levels in plant tissues. A method based on immunoaffinity chromatography (IAC) in combination with LC–MS has also been recently described (Hradecká et al. 2007). Early analytical methods for measuring levels of ABA metabolites employed GC coupled to electron capture detection (GC–ECD; Harrison and Walton 1975; Zeevaart and Milborrow 1976) or flame ionisation detection (FID; Watts et al. 1983) systems. These methods were capable of quantifying PA, DPA, *epi*-DPA and ABA glucose ester (ABAGE) in plant tissues at levels of about ng/g FW. Boyer and Zeevaart (1982) also developed a method for measuring ABAGE, as its tetraacetate derivative, by GC–ECD. In addition, several methods for quantifying ABA and ABAGE by GC–MS in selected ion monitoring (SIM) mode (Netting et al. 1982; Duffield and Netting 2001) and multiple ion monitoring (MIM) mode (Neill et al. 1983) have been published. However, GC–MS is limited to the analysis of volatile compounds, thus methylation of these analytes with diazomethane prior to the analysis is required. Regarding detection techniques following GC separation, ECD permits quantitative analyses of ABA in much smaller samples of plant material than FID. When GC coupled with MS in SIM mode, even much higher sensitivity is then achieved. Further, methods for determining ABA by CE have been published (Liu et al. 2002, 2003). CE has advantages for analysing ABA (as a trace substance in complex plant extracts), but it suffers from low sensitivity in combination with UV detection. This problem can be overcome using either micellar electrokinetic capillary chromatography (MECC; Liu et al. 2002) or laser-induced fluorescence (LIF) detection, both of which provide high sensitivity, but again require derivatisation because ABA is not fluorescent. Therefore, in the second cited study by Liu et al. (2003), ABA was labelled with 8-aminopyrene-1,3,6-trisulfonate via reductive amination in the presence of acetic acid and sodium cyanoborohydride. The resulting conjugate was quantified, with fmol detection limits, and the method was used to analyse ABA in crude tobacco extracts. Recently, LC coupled to MS with soft ionisation techniques (ESI, APCI) has proved to be very powerful for analysing substances in plant extracts since they are often polar, non-volatile, thermally labile and (hence) inappropriate for GC analysis. Due to its high selectivity and sensitivity, LC–MS in multiple reaction monitoring mode (MRM) has also become increasingly popular for analysing ABA and its metabolites (Gómez-Cadenas et al. 2002; López-Carbonell and Jáuregui 2005; Chiwocha et al. 2007; López-Carbonell et al. 2009). Another technique that has been used for quantifying ABA and ABAGE is LC–MS in SIM mode, either directly (Hogge et al. 1993; Schneider et al. 1997) or following several purification steps (Vilaró et al. 2006). Further improvements in the analysis of ABA metabolites have been obtained through use of a UHPLC-based MS method, which is faster, affords higher throughput and is more sensitive than conventional LC–MS (Turečková et al. 2009). The detection limits of the technique were found to be at low picomolar levels for ABAGE and ABA acids in negative ion mode, and femtomolar levels for ABAGE, ABAaldehyde, ABAalcohol and the methylated acids in positive ion mode.

Ethylene

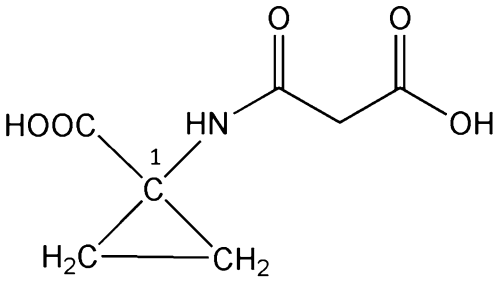
Ethylene is a flammable unsaturated gaseous hydrocarbon (Fig. 5) with a molecular weight of 28.05 g/mol. It has been indirectly used for thousands of years to ripen fruits, for instance via the ancient Egyptian practice of gashing figs (Galil 1948). It seems to have been first described by Becher (1669, Physica Subterranea), identified as a natural plant product by Gane (1934) and shown to influence plant growth and development by Crocker et al. (1935). It is formed essentially in all cells, but often most abundantly in fruits and wounded tissues, diffuses through tissues and is finally released into the surrounding atmosphere. The levels of ethylene produced by plants are low and of the same order as those of other phytohormones. Thus, sensitive methods are essential for its determination. The first methods for ethylene detection, like those for other plant hormones, were based on certain bioassays, mainly because of the lack of instrumental methods at the time. The first was the 'triple response' etiolated pea plant bioassay based on measurement of reductions in stem elongation (Nejlubow 1901) and several others were subsequently developed (Crocker et al. 1932; Addicott 1970; Kang and Rat 1969). However, all the bioassays lack specificity (for instance, propylene, acetylene and butylenes can induce similar responses, albeit at up to a thousand times higher



Ethylene



1-aminocyclopropane-1-carboxylic acid (ACC)



1-(malonylamino)cyclopropane-1-carboxylic acid (MACC)

Fig. 5 Structure of ethylene and its biosynthetic precursors ACC and MACC

concentrations than ethylene) and thus are rarely used now. The development of chromatographic (especially gas chromatography) techniques allowed the identification (by coupling to MS) and quantification of low molecular weight hydrocarbons including ethylene and its biosynthetic precursors 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC), for structures see Fig. 5. All of the mentioned substances of biological interest can be clearly distinguished from other low molecular weight hydrocarbons with high accuracy at approximately 10^{-12} m^3 dm^{-3} in a 1 cm^3 volume (ppb level) of air. The first GC method for ethylene determination was applied to measure this substance from apples (Burk and Stolwijk 1959; Huelin and Kennett 1959). The major drawback of this approach based on thermal conductivity detection (TCD) was a relatively high detection limit of 10–100 μL/L. The introduction of flame ionisation detection (FID) and the photoionisation detector (PID) in 1980s significantly improved the detection limit of ethylene to tens of nL/L levels (Bassi and Spencer 1985; Bassi and Spencer 1989). At the beginning, the ethylene sampling procedure and its subsequent injection into the GC column have been done manually with a gas-tight syringe, which was filled with gas from the headspace of a closed cuvette, in which the plant was enclosed for a few hours (Abeles et al. 1992). For low reproducibility and high time consumption of this system, it has been later replaced by automatic samplers based on concentric rotary valves (Cristescu et al. 2013). To achieve better sensitivity, GC systems can be equipped with preconcentration devices that enable to store the emitted ethylene (Segal et al. 2000). The plants are placed in closed cuvettes and continuously flushed with air. Ethylene is trapped inside a tube containing an appropriate adsorption material (e.g. carbon molecular sieve) and is then released into a smaller volume by heating the adsorbent. In addition to GC and GC–MS (Smets et al. 2003) approaches, photo acoustic laser spectrophotometry (PALS; Cristescu et al. 2008), LC–MS (Petritis et al. 2003) and CE–LIF (Liu et al. 2004) methods for ethylene (or ACC) determination have been published. PALS offers higher detection sensitivity (ppt level) than GC and is highly selective for particular substances. This is disadvantageous in some respects, as the equipment has much narrower applications than GC. However, before use of the GC–MS and CE–LIF methods, the analytes in plant extracts must be modified by derivatisation, which greatly increases time consumption, and the derivatisation procedure has poor reproducibility when concentrations of ACC are low. Recently, methodology based on in vitro measurement of the activity of two key biosynthetic enzymes, 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), as well as ethylene itself, has been reported (Bulens et al. 2011).

Brassinosteroids

Brassinosteroids (BRs) are relatively young group of naturally occurring triterpenoid plant growth substances with hormonal function (Caño-Delgado et al. 2004). More than 70 BR analogues have been identified so far in nearly 60 plant species (Choe 1999). Their common structural feature is a 5α-cholestane skeleton (Fig. 6) and they are divided

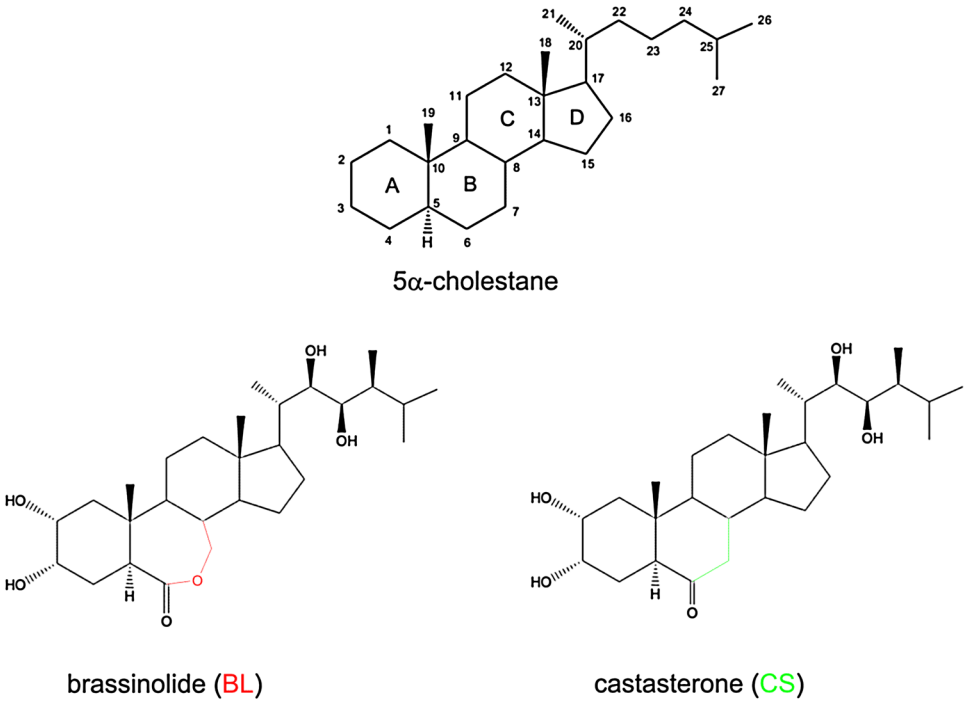


Fig. 6 Structure of 5α-cholestane and biologically active naturally occurring brassinosteroids

into different categories depending on the side chain structure and modifications of the A and B rings. Physiologically, BRs participate with other plant hormones in the regulation of numerous developmental processes, including shoot growth, root growth, vascular differentiation, fertility and seed germination (Fujioka and Sakurai 1997). BRs also have anti-stress effects, i.e. they participate in ameliorative responses to various stresses, such as low and high temperature, drought and infection. Like GAs, they tend to be relatively abundant in reproductive plant tissues, such as pollen, flowers and immature seeds, but their levels are extremely low in vegetative tissues, even compared to those of other plant hormones (fg-pg/g FW).

Initially, immunoassays and bioassays were mainly used for detecting BRs (Takatsuo and Yokota 1999). Some of the bioassays have good sensitivity and are still used for testing the biological activity of BRs, particularly the bean second-internode bioassay (Mitchell and Livingston 1968) and rice lamina inclination bioassay (Maeda 1965), which provide 2×10^{-11} mol and 1×10^{-13} mol detection limits, respectively (Thomson et al. 1981; Wada et al. 1984). Immunological methods such as RIAs and ELISAs have also been used for exploring the distribution of BRs in plant tissues (Horgen et al. 1984; Yokota et al. 1990). RIA was found to be useful for detecting the two most common bioactive BRs, castasterone (CS) and brassinolide (BL) (Fig. 6), with approximately 0.3 pmol detection limits. However, ELISA based on a mouse monoclonal antibody against 24-*epi*brassinolide (*epi*BL) was shown to respond not only to *epi*BL but also to other, non-BR phytosterols (sitosterol, ecdysone). So, this method could not be used for analysing BRs. Swaczynová et al. (2007) subsequently improved the ELISA method using selective polyclonal antibodies against 24-*epi*castasterone (*epi*CS) and successfully detected this substance in *Brassica napus* and *Arabidopsis* tissues. These antibodies cross-reacted with BL and *epi*BL, but not with non-BR plant sterols. Thus, the method was applied for determining BR levels in extracts of tissues from several plant species. In addition, good agreement was found between results obtained using the ELISA method and a simultaneously developed HPLC–MS approach.

Several hyphenated (GC–MS and LC–MS) techniques were also gradually introduced. Since BRs are not volatile they must be derivatised prior to GC–MS analysis (Takatsuo et al. 1982). The standard derivatisation procedure is based on formation of bis-methaneboronates (BMBs) of BRs with vicinal diol groups (e.g. BL and CS). Thus, a disadvantage of this approach is that it cannot be used to analyse BRs lacking this conformation (e.g. teasterone and typhasterol). The detection limits of BMB derivatives are at the sub-ng level. GC–MS has also been used to elucidate structures of new BRs and BR biosynthesis pathways (Fujioka and Sakurai 1997). Liquid chromatography is generally suitable for non-volatile compounds, therefore, BRs can be advantageously analysed using this technique without derivatisation. However, although several LC methods have been published, only one can be used for direct determination of free BRs (Swaczynová et al. 2007). The others still require derivatisation. Gamoh et al. (1996) developed a method based on preparation of naphthaleneboronates,

which is also applicable to teasterone and typhasterol (unlike BMB derivatisation). It has a reported detection limit of 2 ng and was applied to analyse BRs in *Cannabis sativa* seeds. Another LC method provides 125 attomole detection limits for dansyl-3-aminophenylboronate derivatives of BRs in highly laboriously purified extracts (57 g) of 24-day-old *Arabidopsis* plants grown in vitro. (Svatoš et al. 2004). Recently, two other LC–MS methods for preparing and analysing boronate derivatives of BRs have been reported (Huo et al. 2012; Ding et al. 2013), but the starting amount of tissue (*Arabidopsis*) used in the cited studies was still extremely high: 1 or 2 g FW.

Jasmonates

Jasmonic acid (JA) and its metabolites, collectively called jasmonates (JAs), are cyclopentanone compounds (Fig. 4) that share remarkable structural and functional properties with prostaglandins found in animals (Wasternack and Kombrink 2010). In the 1990s, JAs were proposed to be stress-related compounds (Farmer and Ryan 1990; Parthier 1991) that accumulate in plants in response to various stresses, such as wounding or pathogen attack (Creelman et al. 1992), in plant tissues or cell cultures treated with fungal elicitors (Müller et al. 1993), and tissues subjected to abiotic stressors such as UV radiation, low and high temperatures, osmotic stress and ozone exposure (Parthier et al. 1992). JAs seem to occur in most organs of most plant species (Wasternack and Hause 2013). Their *in planta* concentrations, which can be determined by various methods, are comparable to those of other plant hormones, ranging from ng to μg/g FW, depending on the plant tissue, species, developmental stage and both environmental and physiological conditions (Wilbert et al. 1998). The major physiologically active jasmonates are reportedly (−)-JA, methyl jasmonate (MeJA), and conjugates of (−)-JA with the amino acids isoleucine (JA-Ile), valine (JA-Val), and leucine (JA-Leu) (Sembdner and Parthier 1993). JA-amino acid conjugates are constitutively produced in plant tissues and their levels increase upon osmotic stress (Kramell et al. 1995). In plant–herbivore interactions, JA-amino acid conjugation is necessary for JA activation, and (−)-JA-Ile is the bioactive form of the hormone (Staswick and Tiryaki 2004; Fonseca et al. 2009). MeJA plays important roles as a fragrant volatile compound, particularly in plant–plant interactions, in which it acts, in concert with other volatile substances emitted from the plants, as an aerial signal for communication with their environment (Pichersky and Gershenzon 2002). Its synthesis is induced by various external stimuli, such as adverse weather conditions, and attacks by herbivores or pathogens (Paré and Tumlinson 1999). Another JA metabolite, which is highly active in plant–insect interactions, is *cis*-jasmone (Birkett et al. 2000; Bruce et al. 2008).

Key considerations in the analysis of jasmonates are that JA and its conjugates are non-volatile, while MeJA and *cis*-jasmone are volatile. GC–MS is the most frequently used approach for quantifying JA, but as JA is not volatile it must first be derivatised, for instance by preparation of pentafluorobenzyl ester derivatives (Müller and Brodschelm 1994). This provides high sensitivity, but requires an elaborate preconcentration procedure. Another GC–MS-based technique for JA quantification has been described (Engelberth et al. 2003), in which the only purification step is collection of derivatised JA on a polymeric adsorbent. Nevertheless, these time-consuming steps still seriously limit the number of samples that can be processed per day. MeJA can be successfully quantified directly by GC with FID or MS detection after concentration by solid-phase microextraction (SPME) on fused silica fibre coated with a polymeric sorbent (Meyer et al. 1984). The reported detection limit of this method is 1 ng/injection, sufficiently low for detecting MeJA in plant tissues at levels between 10 and 100 ng/g DW (Müller and Brodschelm 1994; Wilbert et al. 1998).

Due to the polarity and non-volatility of JA most researchers use LC-based methods for its analysis. Anderson (1985) described an HPLC assay for the simultaneous determination of ABA and JA in plant extracts, following derivatisation (of both growth regulators) with a fluorescent hydrazide to obtain stable fluorescent products—dansyl hydrazones. This procedure allows detection of both hormones at low pmol levels. The method was demonstrated using extracts of several different tissues of soybean (*Glycine max*), snap beans (*Phaseolus vulgaris*), lima beans (*Phaseolus lunatus*) and broccoli (*Brassica oleracea*). However, only 20 % of the JA was converted to the corresponding ester during the derivatisation procedure. Fluorescent labelling usually affords great sensitivity for detecting the resulting derivatives (approx. 10^{-17} mol) and thus prompted other researchers to optimise this kind of derivatisation to introduce fluorophores into the chemical structure of JA to monitor it after separation by either HPLC (Kristl et al. 2005; Xiong et al. 2012) or CE (Zhang et al. 2005). However, the highest selectivity and sensitivity for JA determination can be currently achieved using MS/MS in MRM mode (Tamogami and Kodama 1998; Wilbert et al. 1998; Segarra et al. 2006). Another of JA's key physicochemical properties is amenability to oxidation, which was recently exploited for its electrochemical detection following LC (Xie et al. 2012). The method was successfully applied to analyse endogenous JA in wintersweet flowers and rice florets with a detection limit of 10^{-8} mol/L. To study physiological process in plants under various stresses, many researchers also monitor levels of precursors in the

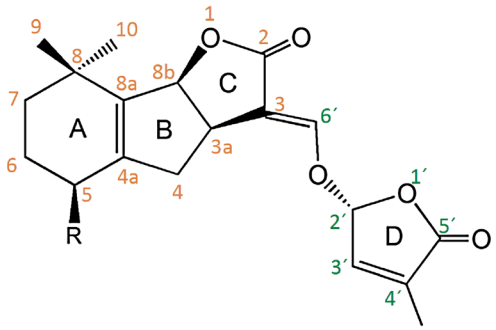
JA biosynthetic pathway (especially 12-oxo-phytodienoic acid, OPDA) and JA metabolites. For these studies, LC–MS/MS is the method of choice (Radhika et al. 2012; VanDoorn et al. 2011). Similarly, stress resistance investigators (profiling the main stress response actors such as JAs, ABA and salicylic acid) generally use LC–MS/MS methods, which have been accelerated by coupling UHPLC, rather than conventional HPLC, systems to tandem mass spectrometers (Flors et al. 2008; López-Ráez et al. 2010; Balcke et al. 2012). This strategy also increases sensitivity, allowing successful quantification of target stress hormones in milligram quantities of plant tissue samples.

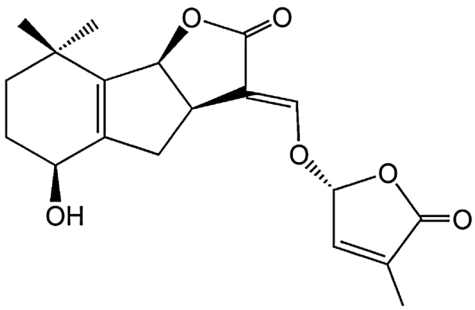
Salicylic acid

Salicylic acid (SA; Fig. 4) is an endogenous signalling molecule that is predominantly active in plant immune responses to avirulent pathogens, but like other phytohormones it is also involved in the regulation of several developmental processes, especially flowering (Singh et al. 2013). In most plants, pathogen attack, insect feeding and other kinds of physical wounding trigger both local and systemic resistance, mediated by the accumulation of defence-related proteins at sites of infection/damage and healthy tissues, respectively (Hammond-Kosack and Jones 2000). On the molecular level, accumulation of SA in cells leads to the release of NPR1 protein, activation of TGA1 and TGA2 transcription factors and expression of pathogenesis-related proteins (Pieterse and Van Loon 2004). To elucidate their signalling roles, SA and its metabolites (including methylsalicylate, salicylic acid glucoside and salicylic acid glucosylester) must be precisely quantified in plant tissues by robust, sensitive analytical methods. HPLC with fluorescence detection has been successfully used for quantifying SA, following a complex purification procedure (Meuwly and Metraux 1993), in cucumber (*Cucumis sativus* L.) seedlings infected by *Pseudomonas lachrymans* on the first leaf. Free SA contents increased locally in the infected leaf and systemically in the second leaf to 33-fold and 4.2-fold higher than detection limits, respectively, while remaining undetectable in controls. Recently, another HPLC method with fluorescence detection has been reported for quantitative analysis of SA in tobacco leaf tissues (Verberne et al. 2002). This methodology increased SA extraction recovery from plant tissues by reducing SA sublimation during purification via the addition of a small amount of HPLC eluent, resulting in recoveries in the range of 71–91 % for free SA and 65–79 % for acid-hydrolysed SA.

However, fluorometric analysis cannot fully distinguish SA and its metabolites from other plant components, particularly simple phenolics and phenylpropanoids, which might be present in infected plants and participate in disease resistance. Partly for this reason, methods allowing their precise, accurate determination based on analyses of their molecular masses and specific daughter fragments (tandem MS) or other analyte-specific approaches have been developed. Initially, a method based on electrospray tandem MS coupled to capillary LC was introduced to detect SA (together with JA and MeJA) in extracts of fresh poplar leaves (Wilbert et al. 1998). In addition, a bacterial biosensor that is highly specific for SA, methyl-SA and the synthetic SA derivative acetylsalicylic acid was recently shown to be suitable for quantifying SA in crude plant extracts (Huang et al. 2005). Following increases in its throughput, this approach was successfully applied for genetic screenings for SA metabolic mutants and characterising enzymes involved in SA metabolism (Defraia et al. 2008; Marek et al. 2010). Another LC–MS/MS approach has been applied to study SA and JA levels in cucumber cotyledons under biotic stress induced by the necrotrophic pathogen *Rhizoctonia solani* (Segarra et al. 2006). An LC–MS method has also been compared to a capillary electrophoresis (CE) technique, and used to study SA and related phenolics in wild-type *Arabidopsis* plants and two lines with mutations affecting SA accumulation in response to two avirulent bacterial strains (Shapiro and Gutsche 2003). Furthermore, in efforts to elucidate SA metabolism Pastor et al. (2012) developed an LC–MS/MS method that enabled them to identify two conjugates: salicylic glucosyl ester (SGE) and glucosyl salicylate (SAG). Their results also revealed that SA and its main glucosyl conjugates accumulate in *Arabidopsis thaliana* in a time-dependent manner, in accordance with the up-regulation of SA-dependent defences following *Pseudomonas syringae* infection. In addition to SA signalling, Belles et al. (1999) found that gentisic acid, a product of SA hydroxylation, is a complementary pathogen-inducible signal that is essential for accumulation of several antifungal pathogenesis-related proteins in tomato. Both gentisic and salicylic acids were quantified in SA-treated chamomile by a rapid UPLC-MS/MS method originally developed for analysing hydroxybenzoates and hydroxycinnamates in beverages (Gruz et al. 2008; Kovacik et al. 2009). SA was accurately quantified using deuterium-labelled internal standards of salicylic (3,4,5,6-[$^2H_4$]) and 4-hydroxybenzoic (2,3,5,6-[$^2H_4$]) acids to account for ESI–MS signal trends, matrix effects and potential extraction losses.

GC–MS has also been used to quantify SA, after derivatisation (to enhance volatility and sensitivity) of the carboxylic acid with diazomethane to form SA methylester (Scott and Yamamoto 1994). The disadvantage of this method is that it requires elaborate sample preparation, from ca. 1 g FW of tissue, including anion exchange and preparative HPLC. In 2003, Engelberth and co-workers introduced a method for SA and JA analysis based on collecting





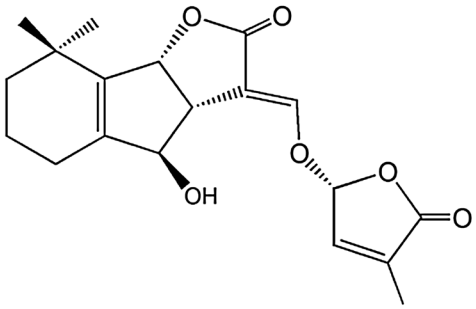


Fig. 7 Natural strigolactones—basic structure and the examples

derivatised and volatilised compounds on a polymeric adsorbent and GC-positive ion chemical ionisation-MS using only milligrams of plant tissue. This method was later used to explore changes in the metabolic profiles of SA, cinnamic acid, JA, IAA, ABA, unsaturated C18 fatty acids, 12-oxo-phytodienoic acid, and the phytotoxin coronatine in *Arabidopsis* infected by *P. syringae* (Schmelz et al. 2004). However, this approach also requires both purification and derivatisation steps.

Strigolactones

Strigolactones (SLs) are the most recently described class of plant hormones. They were originally regarded as a family of carotenoid-derived plant secondary metabolites (Xie et al. 2010) with roles in signalling between organisms (allelochemicals). Initially, they were of interest due to their action as seed germination stimulants of the root parasitic allelopathic weeds *Orobanche* and *Striga* (Cook et al. 1966, 1972). Seeds of these parasites germinate only when they perceive chemical signals produced by and released from the roots of other (host or nonhost) plants. This germination-inducing activity of SLs was the basis of a sensitive SL bioassay using *Striga* and *Orobanche* seeds (Joel et al. 1995), and very recently a fast convenient method for determining the germination rate of parasitic weeds seeds has been reported (Pouvreau et al. 2013). In 2008, SLs were classified as a new group of plant signalling molecules with endogenous hormonal activity due to involvement in the inhibition of shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Earlier it was also found that they are able to stimulate hyphal branching in mycorrhizal fungi (Akiyama et al. 2005). To date, more than 20 compounds in this family of sesquiterpene lactones have been identified from root exudates of various plant species (Tsuchiya and McCourt 2009; Xie et al. 2010). The most well-known SLs are strigol and orobanchol (Fig. 7). Structurally, all natural SLs contain a tricyclic lactone skeleton (cycles A, B and C) bound to a butenolide moiety (ring D) via an enol ether bond. The A and B rings bear various substituents (Zwanenburg et al. 2009).

A pioneering GC–MS approach for analysing SLs in plant samples was introduced by Yokota et al. (1998). However, this method required time-consuming purification of extracts and TMS derivatisation. Furthermore, some SLs partially decomposed during the procedures. An approach for analysing strigol heptafluorobutyrate derivatives obtained from extracts of *Striga asiatica* host plants (maize and proso millet) was subsequently developed (Siame et al. 1993), based on electron impact (EI) and both positive and negative chemical ionisation (CI) mass analyses of HPLC fractionated samples. This method avoided some of the previous problems, but the purification procedure

was still extremely tedious and labour intensive. Another two approaches for analysing SLs, based on LC–MS/MS, have been published, with detection limits (in MRM mode) ranging between 0.1 and 1 pg/μL of SLs (Sato et al. 2003, 2005). Most recently, Xie et al. (2013) reported an LC–MS/MS procedure allowing structural elucidation of 11 SLs in root exudates of tobacco and detection of four SLs in rice. Notably, no SL profiling has been reported to date.

Hormone profiling

Since it is becoming increasingly evident that hormones do not act separately, but have highly interactive physiological effects and mutually affect each other's biosynthesis and metabolism, there is increasing interest in 'hormone profiling', i.e. analysing hormones of several classes (together with their precursors and metabolites) in the same plant tissue simultaneously. This greatly increases analytical complexity as it requires methodology capable of quantitatively detecting chemically and structurally diverse substances rather than a single targeted group of plant hormones. Since many plant hormones are acidic, published methods have often focused on these classes of compounds (Müller et al. 2002; Schmelz et al. 2003; Durgbanshi et al. 2005). Hormone profiling was first successfully applied, by Chiwocha et al. (2003), in a study of thermodormancy where four CKs and 10 acidic plant hormones (IAA, ABA, ABAGE, 7′OH-ABA, PA, DPA and four GAs) in 50–100 mg DW extracts of lettuce seeds were all measured by LC–MS, using a single purification step and a 40 min chromatographic gradient. Pan et al. (2008) subsequently developed an LC–MS/MS technique (requiring no purification or derivatisation) for simultaneous quantification of 17 plant hormones including auxins, CKs, ABA, GAs, JAs, salicylates and corresponding methyl esters in crude extracts of samples (50–100 mg) of Arabidopsis plants that had been mechanically wounded or challenged with the fungal pathogen *Botrytis cinerea*. Limits of quantification reportedly ranged from 0.01 to 10 pg/g FW. In addition, a fast LC–MS/MS method combined with an automatic liquid handling system for SPE was recently used for simultaneous analysis of 43 plant hormone substances, including CKs, auxins, ABA and GAs in the rice GA-signalling mutants *gid1-3*, *gid2-1* and *slr1* to study relationships between changes in gene expression and hormone metabolism (Kojima et al. 2009). To enhance sensitivity, a nanoflow-LC–MS/MS approach has also been used to detect 14 plant hormones, following a two-step purification procedure, in extracts of *Arabidopsis* and tobacco seedlings (Izumi et al. 2009). The limit of detection was found to be in the sub-fmol range for most studied analytes. However, this method failed for the acidic plant hormones, especially GAs. Capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) has also been applied in profiling, to simultaneously determine plant hormones containing carboxyl groups, including GA_3, IAA, indole butyric acid (IBA), 1-naphthalene acetic acid (NAA), 2,4-dichloro-phenoxy acetic acid (2,4-D), ABA and JA in crude extracts of banana samples (500 mg) without further purification (Chen et al. 2011). Finally (for this summary), 20 plant growth substances (including IAA, ABA, CKs and structurally related purines) have been determined in single chromatographic runs (Farrow and Emery 2012). The methodology involved extraction from 100 mg samples of *Arabidopsis thaliana* leaves, purification and analysis by conventional HPLC with a fused core column. QTRAP mass analyzer has been utilised here for detection of selected analytes. Reported detection limits ranged from 2 pmol for zeatin-9-glucoside to 750 pmol for IAA. High-resolution MS (HR-MS) is not yet widely used for quantitative analysis of plant hormones, but will probably soon be employed for their routine quantification. An HR-MS approach has already been applied to identify and quantify a large number of endogenous phytohormones in tomato fruits and leaf tissues (Van Meulebroek et al. 2012). The cited authors selected eight phytohormones—GA_3, IAA, ABA, JA, SA, zeatin (not mentioned if *trans*- or *cis*-), BAP and *epi*BL—as representatives of the major hormonal classes, and applied a simple extraction procedure followed by UHPLC-Fourier Transform Orbitrap MS separation and detection for hormonally profiling 100 mg FW samples of the tomato tissues. The samples were extracted in methanol:water:formic acid (75:20:5) over 12 h, ultracentrifuged, then injected directly into an UHPLC system equipped with C_{18} column of 2.1 × 50 mm diameter (particle size 1.8 μm) and coupled to a benchtop Orbitrap mass spectrometer, equipped with a heated electrospray ionisation source (HESI), operating in both positive and negative modes. This technique provided detection limits of the analytes ranging from 0.05 to 0.42 pg/μL. Moreover, full mass scans by the Orbitrap MS provided a dataset including information on hundreds of matrix compounds. Therefore, this metabolomic profiling approach might lead to the discovery of compounds with no previously known hormonal activities or roles in plants. However, although HR-MS provides opportunities to use narrow mass windows to exclude interfering matrix compounds and selectively analyse substances (Kaufmann 2012), Van Meulebroek et al. (2012) used a relatively broad mass window of 5 ppm, to ensure that no compounds would be completely undetected.

Conclusion and perspectives

Analysis of plant hormones is very challenging because they have extremely wide ranges of physicochemical

properties, and plant tissues contain trace quantities of hormones together with thousands of other substances at far higher levels. However, there have been great advances in analytical techniques used in diverse "life sciences" during the last decade. LC–MS has become the most versatile, rapid, selective and sensitive technique available for identifying and quantifying small molecules (Pan et al. 2008; Van Meulebroek et al. 2012). Thus, it is replacing all other approaches in plant hormone analysis. New technologies based on a unique ion transfer device designed to maximise ion transmission from the source to the mass analyzer, could further improve the sensitivity (typically the primary concern) of phytohormone measurements. Typical gains obtained using such device include generally 25-fold increases in peak areas and 10-fold increases in signal-to-noise ratios, which are highly valuable for phytohormone quantifications. The next challenge could be to develop robust techniques for extending the breadth of profiling, including more phytohormone precursors and metabolites, as well as those of other signalling molecules in plants. LC–MS/MS methods may be particularly useful for this as they afford capabilities for simultaneously quantifying metabolites with diverse properties of both single and multiple phytohormone groups.

Even with further anticipated advances it will be extremely challenging to quantify all phytohormones and related compounds in a single LC–MS/MS run due to their high chemical diversity and the inherent difficulty in distinguishing numerous metabolites that may have very similar chromatographic properties, share the same mass and yield very similar fragments. The extremely high levels of matrix compounds typically present in plant extracts compound the problem. However, additional orthogonal separation techniques have been recently introduced that provide high selectivity, further improve spectral quality, enhance the quality of acquired datasets and facilitate their interpretation, thus surmounting some of the difficulties. Notably, ion mobility separation (Eugster et al. 2012) and MS^E have been combined in a powerful new approach called high definition mass spectrometry (Sotelo-Silveira et al. 2013), which could generate more precise datasets from explorations of endogenous phytohormone levels and their changes during developmental processes in plants. The recent progress in analytical MS technologies could also enable tissue- and cell-specific quantifications as well as analyses of levels of multiple hormones in single plant cells or subcellular compartments.

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