# GIBBERELLINS – TERPENOID PLANT HORMONES: BIOLOGICAL IMPORTANCE AND CHEMICAL ANALYSIS

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Gibberellins (GAs) are a large group of diterpenoid carboxylic acids, some members of which function as plant hormones controlling diverse aspects of growth and development. Biochemical, genetic, and genomic approaches have led to the identification of the majority of the genes that encode GA biosynthesis and deactivation enzymes. Recent studies have shown that both GA biosynthesis and deactivation pathways are tightly regulated by developmental, hormonal, and environmental signals, consistent with the role of GAs as key growth regulators. In this review, we summarize our current understanding of the GA biosynthesis and deactivation pathways in plants and fungi, and discuss methods for their qualitative and quantitative analysis. The challenges for their extraction and purification from plant tissues, which form complex matrices containing thousands of interfering substances, are discussed.

**Keywords**: Gibberellins; Biosynthesis; Signaling; Profiling; Extraction; Purification; Mass spectrometry; Liquid chromatography.



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## 1. INTRODUCTION

Plant tissues contain various signalling molecules, which act in low concentrations to regulate growth and development and play a crucial role in most physiological processes. These substances, known as plant hormones or, less widely, "phytohormones" include the following main groups: auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids, jasmonates and ethylene. Recently, a novel group of compounds named the strigolactones[1], were also shown to possess growth-regulating activity, and it is likely that other groups of plant hormones will be discovered in the future.

Gibberellins (GAs) are a class of diterpenoid carboxylic acids, which include biologically active compounds that are widely distributed throughout the plant kingdom and are also produced by some fungal and bacterial species. The name of this group of plant hormones was derived from the pathogenic fungus *Gibberella fujikuroi* which causes a disease termed "foolish seedling" or bakanae (in Japanese) in rice (*Oryza sativa*). The disease causes rice plants to grow extremely fast, become spindly and pale as well as sterile. To date, 136 naturally occurring GAs have been characterised from higher plants, fungi and bacteria (www.plant-hormones.info/ga1info.htm).

The biologically active GAs play an intrinsic role in many physiological processes throughout the whole plant life cycle. Their functions include induction of germination by breaking of seed dormancy[2–4], stimulation of hypocotyl elongation[5], flower initiation, induction of stem elongation via enhanced cell division and elongation. They also induce maleness in dioecious flowers (sex expression) and support flower organ development, particularly of the stamens, in which they promote filament elongation, anther development as well as the development, germination and tube growth of the pollen[6,7]. Gibberellins can also cause parthenocarpic (seedless) fruit development in the absence of fertilisation and delay of senescence in leaves and citrus fruits[7].

## 2. GIBBERELLIN CHARACTER AND SIGNALLING

All natural GAs possess a tetracyclic (four-ringed) *ent*-gibberellane skeleton (containing 20 carbon atoms), or a 20-nor-*ent*-gibberellane skeleton (con-

tains only 19 carbon atoms, carbon-20 is missing), i.e. in terms of number of carbons, GAs can be divided into two groups, $C_{19}$-GAs (e.g. $GA_9$) and $C_{20}$-GAs (e.g. $GA_{12}$). The prefix *ent* refers to the fact that the skeleton is derived from *ent*-kaurene (Fig. 1), a tetracyclic hydrocarbon that is enantiomeric to the naturally occurring compound, kaurene.

No plant produces all the known GAs and these GAs are not all equally biologically active; some are precursors and some are deactivation products of the biologically active GAs. $C_{20}$-GAs with a carbon atom attached to $C_{10}$ are considered precursors of $C_{19}$-GAs [3]. Among features crucial to bioactivity are the hydroxy group on C-3 and the carboxyl group on C-6, lack of which also leads to loss of activity[8]. These groups enhance binding to the receptors through interaction with polar amino-acid residues while a hydroxy group on C-2 decreases binding affinity[9,10]. Consequently, hydroxylation on C-2 causes GA inactivation and is an important mechanism for regulating the concentration of GAs in angiosperms (flowering plants)[11].

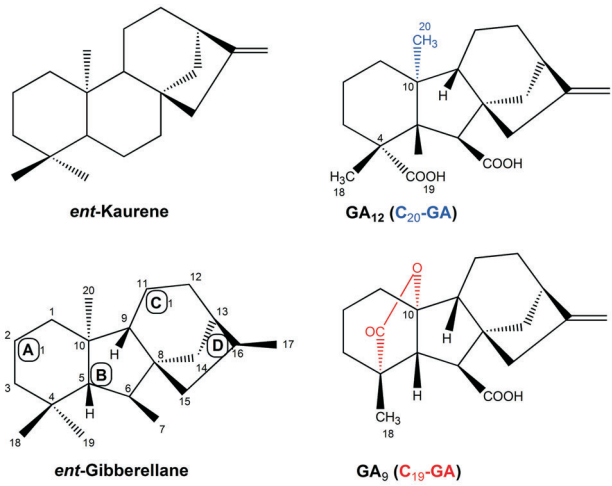


FIG. 1
Structures of *ent*-kaurene, *ent*-gibberellane and an example of $C_{19}$ and $C_{20}$ gibberellins

### 2.1. Gibberellin Biosynthesis/Metabolism

The outline of the GA-biosynthetic pathways has been described in many reviews[12–15]. Gibberellins are formed from geranylgeranyl diphosphate (GGDP), the common $C_{20}$ precursor for all diterpenoids[16]. GGDP can be formed from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate

(DMAPP) either via the deoxyxylulose phosphate (DOXP) pathway, or via the mevalonic acid (MVA) pathway. The DOXP pathway operates in the plastids of vegetative tissues while the MVA pathway occurs in the cytosol. Therefore, certain equilibrium of IPP must exist between these two cell compartments[16]. The contribution of these two pathways varies between different tissues according to the type of plastid and to the permeability of its membrane. Nevertheless, the DOXP pathway was shown to provide the majority of the isoprene units to GAs in the seedlings of the model plant *Arabidopsis*, whereas there is a minor contribution from the cytosolic MVA pathway[16]. The GA-biosynthetic pathway proceeds in three stages, each of which takes place in a different cellular compartment (Fig. 2). Basically, *ent*-kaurene, a tetracyclic hydrocarbon, is synthesised in two steps from GGDP in plastids with involvement of two enzymes, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase[17,18]. *ent*-Kaurene is then sequentially oxidized to yield the first-formed GA, $GA_{12}$ and its C-13 hydroxylated analogue $GA_{53}$. The oxidation of *ent*-kaurene to $GA_{12}$ is catalyzed by two cytochrome P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid hydroxylase (KAO). KO was found to be present in the outer membrane of the plastid and KAO is located in endoplasmatic reticulum[18]. The third stage of the pathway, in which $GA_{12}$ is converted to bioactive $GA_4$, occurs in the cytosol. This conversion comprises oxidations on C-20 and C-3 by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), respectively. GA20ox catalyzes the oxidation of C-20 from a methyl group to an aldehyde followed by its removal to produce $C_{19}$-GAs. For example, $GA_{12}$ and $GA_{53}$ are oxidised by GA20ox to the $C_{19}$-GAs $GA_9$ and $GA_{20}$, respectively. The introduction of a 3β-hydroxy group by GA 3-oxidase (GA3ox) converts these $C_{19}$ inactive precursors into bioactive GAs ($GA_4$ and $GA_1$, respectively)[19].

In order to enable effective regulation of the concentration of the bioactive hormone in plant cells, it is necessary that they are deactivated, for which several mechanisms are known. The major route is via 2β-hydroxylation of $C_{19}$- as well as $C_{20}$-GAs by GA 2-oxidases (GA2oxs)[20–23]. An alternative mechanism was discovered in rice, in which epoxidation of the 16, 17-double bond of non-13-hydroxylated GAs, including $GA_4$, $GA_9$ and $GA_{12}$, was described[24]. Developing seeds of Arabidopsis were found to contain GA-specific methyl transferases, which converted the C-6 carboxyl group to biologically inactive methyl esters[25]. A further, possibly reversible, mechanism for GA deactivation is formation of glucose conjugates, to form either ethers or esters[26]. The GA-*O*-glucosyl ether (GA-*O*-Glc) results from conjugation to glucose via a hydroxy group, while the GA-glucosyl ester is

a product of the conjugation via the 7-carboxyl group. The most common sites for –O-Glc conjugation are carbons C-2, C-3 and C-13 within the GA molecule.

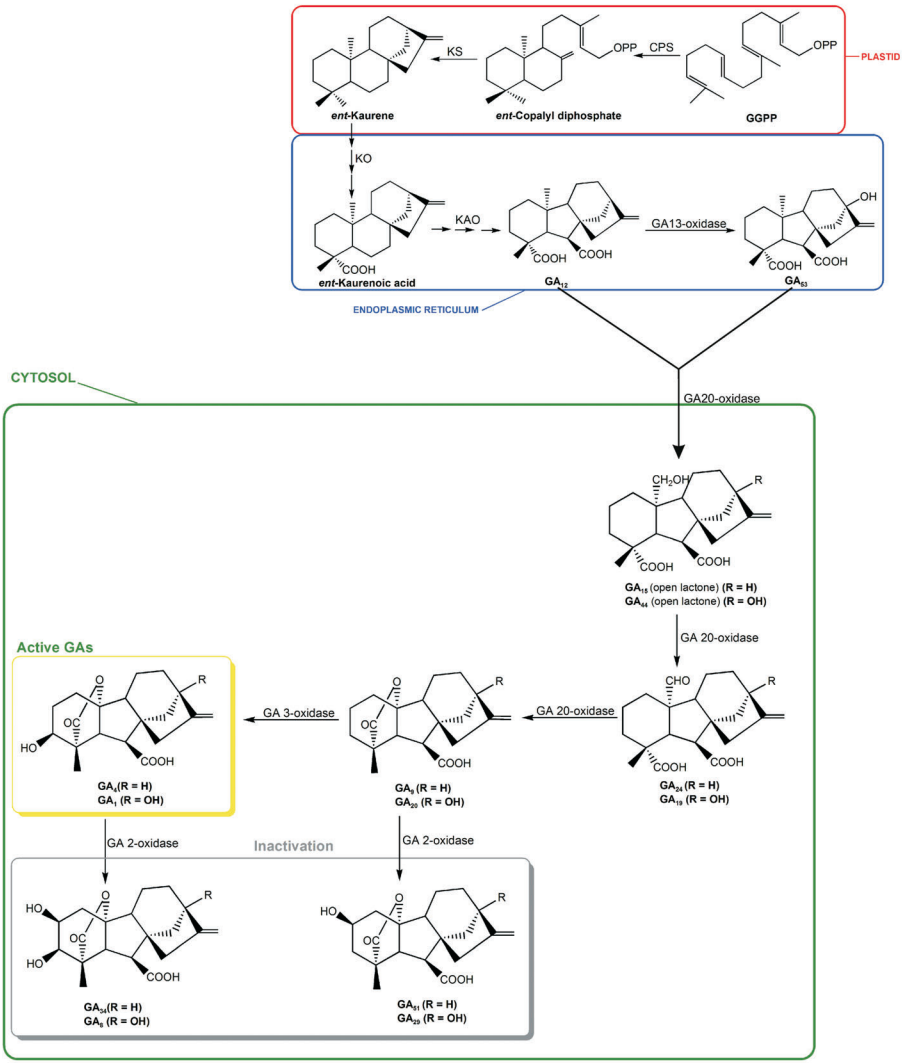


FIG. 2
Scheme of gibberellin biosynthesis and metabolism

Recently, after identifying genes encoding GA biosynthesis enzymes from fungi (*G. fujikuroi*), remarkable differences in GA biosynthesis pathways and enzymes between plants and fungi were revealed. In plants, two separate terpene cyclases (CPS and KS) catalyse the synthesis of *ent*-kaurene from GGPP (Fig. 2), whereas in fungi a single bifunctional enzyme (CPS/KS) is involved in these two reactions[27–29] (Fig. 3). A similar role to that of KOs and KAOs in plants was found to be played by the fungal P450s. However, these are not closely related to the plant enzymes in amino acid sequence. No-

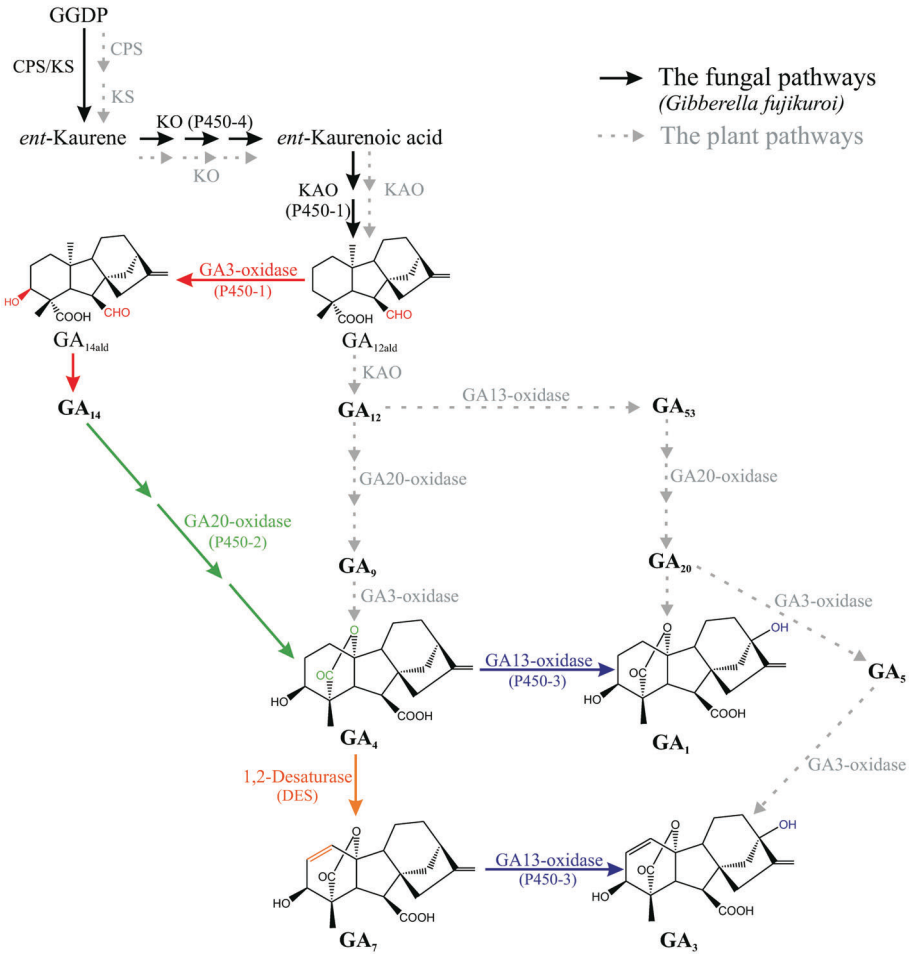


FIG. 3
Scheme of the gibberellin biosynthetic route in the fungus *Gibberella fujikuroi*; comparision to the plant pathways

ticeably, P450-1 has 3β-hydroxylase activity in addition to KAO activity and forms $GA_{14}$ (Fig. 3) which is further converted to GA4 by another P450, P450-2 [30]. $GA_4$ is then desaturated to $GA_7$ and finally hydroxylated to $GA_3$ by P450-3 [31]. These significant differences in the GA biosynthesis pathways between higher plants and fungi clearly indicate that they have evolved independently.

### *2.2. Gibberellin Signal Transduction and Feedback Regulation*

Gibberellin function by repressing the action of DELLA proteins (DELLAs), which are signalling components belonging to the family of transcriptional regulators (transcription is the process of creating an equivalent RNA copy of a sequence of DNA). The N-terminal domain of DELLAs possess highly conserved sequence motifs, one of which includes the sequence of five amino acids aspartic acid (D), glutamic acid (E), leucine (L), leucine (L), and alanine (A), from which the name "DELLA" proteins is derived. DELLAs interact with transcription factors (a substance, such as a protein, that regulates the transcription of a gene). For example, DELLAs in Arabidopsis suppress the action of the light-regulated transcription factors PIF3 and PIF4, which promote hypocotyl growth.

DELLAs are involved in the maintenance of GA homeostasis and in some cases act as points of convergence between GA and other signalling pathways. They are involved in cross talk between GA and some other plant hormones, included auxins. GAs function by initiating the degradation of DELLAs by the 26S proteasome (very large protein complex inside the nucleus and the cytosol of all eukaryotes; the main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds). DELLAs are targeted for degradation by polyubiquitination via an SCF E3 ubiquitin (Ub) ligase, a reaction which is stimulated by GA [18,32]. A nuclear soluble GA receptor, Gibberellin insensitive dwarf1 (GID1)[32], shows a similarity of primary structure to that of the hormone-sensitive lipases (HSLs)[33,34]. In the presence of GAs, GID1 binds to DELLAs and induces their association with the SCF E3 Ub ligase[35]. The structure of the receptor was described for the first time simultaneously by Murase et al.[9] and Shimada et al.[10]. GA was shown to bind in a pocket of GID1, which contains a loose strand at its amino-terminal end that associate with the hydrophobic surface of the bound GA, so covering the pocket like a lid (Fig. 4). The DELLA protein interacts with the upper surface of the lid, and Murase et al.[9] proposed that this association may cause a change in the shape of the DELLA protein, which allows it

to interact with the ubiquitin ligase. Therefore, GA functions as an allosteric activator of GID1, leading to the structural changes that allow the receptor to associate with DELLAs, but it does not interact directly with the DELLAs themselves[36]. The GA-GID1-DELLA molecular mechanism enables plants to respond to the GA signals and thereby modify their growth and development in response to environmental changes.

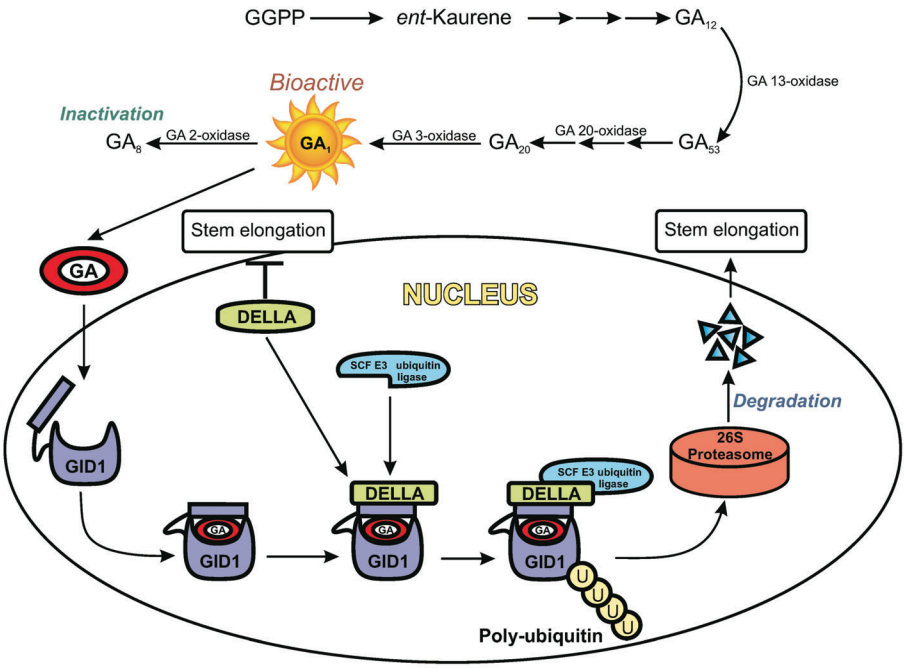


FIG. 4
Gibberellin signal transduction and feedback regulation

### 3. METHODS FOR ANALYSIS OF GIBBERELLINS[37,38]

In common with most other classes of plant hormones, the concentrations of GAs in plant tissues can be extremely low (in general ng $g^{-1}$ of fresh weight), especially in vegetative plant organs (roots, stems and leaves). The reproductive organs (such as seeds) often contain $10^3$ times as much GA as vegetative tissue. Thus, GA analysis requires very sensitive methods for their detection. Moreover, analytical procedures used to investigate endogenous GAs must be able to distinguish a GA from the huge amounts of other plant components. As a consequence of the low concentration of GAs in

plant tissues, tedious and labour intensive preconcentration steps are usually necessary in order to remove interfering substances (e.g. lipids, proteins and pigments) from the crude extracts and yield them sufficiently pure for the final analysis. The choice of extraction and purification method depends not only on the analyte, but also on the type of analysis to be performed and the analytical equipment available. In case of full chemical characterisation of a previously unknown compound (qualitative analysis), much more extensive purification of relatively large amounts of pure substance is needed than, for instance, for the quantitative analysis of known GAs. Rapid and simple procedures with minimal losses are required for the routine high-throughput quantitative analyses of large number of samples. No universal method exists for the analysis of GAs in plant tissue. However, modern analytical techniques (such as LC-MS) are sufficiently sensitive and selective to measure GAs at low concentrations provided steps are taken to eliminate potential interference from contaminants.

GAs cover a broad range of polarities and the only property they share is that, as tetracyclic diterpenoid carboxylic acids, they behave as weak organic acids with $pK_a \approx 4.0$. They display no spectral characteristics such as fluorescence or UV absorption (only below 220 nm) that might easily distinguish them from other organic acids. Some GAs are highly oxidized molecules with many functional groups, and may be relatively labile, especially in aqueous solutions at extreme pH and at elevated temperatures. Under alkaline conditions, 3β-hydroxyGAs undergo a reversible retro-aldol rearrangement resulting in epimerisation, 1,2-dehydro $C_{19}$- GAs, such as $GA_3$, isomerize to the 19,2β-lactones with a shift of the double bond. This rearrangement may also occur in the heated injection port of a gas chromatograph. Drastic heating in aqueous solutions at temperature over 100 °C may lead to their complete degradation. Therefore, extraction and purification procedures should be performed within the range pH 2.5–8.5 and solutions containing GAs (especially aqueous ones) should be handled at temperatures below 40 °C. To avoid aerial oxidation of some of GA precursors, extracts are best stored at –20 °C.

Prior to extraction, plant material is homogenised either by grinding (gramme amounts) in cold extraction medium using knife homogenizers, or by grinding freeze-dried plant tissue with a mortar and pestle followed by adding an appropriate solvent to the ground material. Very small amounts of plant material (mg) can be also homogenised directly in plastic microtubes (1.5 ml) with extraction solvent by adding carbide beads into the tube and placing them into the holder of an electric mill, which grinds the plant material for a defined time at a given frequency. Plant material

should be kept cold during the entire process of homogenisation to avoid enzymatic or chemical degradation of the GAs.

### *3.1. Extraction and Purification*

The efficiency of GA extraction from plant tissue depends on its subcellular localisation and the extent to which it is associated with phenolics, lipids or proteins; it also varies with the polarity of the molecule. The solvent used has to extract the analyte efficiently, whilst the quantity of interfering substances extracted should be as low as possible. Solvents such as cold methanol, aqueous methanol (80% v/v), aqueous methanol acidified with formic acid (0.05% v/v)[39] or mixture of isopropanol:glacial acetic acid[40] are often used for extraction of GAs. Acetone is occasionally used instead of methanol[24] but the formation of acetonides with germinal diols can be observed[41]. Compensation for analyte losses during the sample purification procedure can be achieved by addition of internal standards (usually stable isotope labelled GAs) to the plant tissue extracts. The extraction period takes usually several hours (2 h, overnight or 24 h) to allow the GAs to extract into the medium and to establish isotope equilibrium between the endogenous compound and added internal standard. The risk of breaking down the endogenous GAs during longer duration extractions is minimised by performing the extraction at low temperature (4 °C).

Nowadays, purification of plant extracts is usually achieved by one of two main methods. The first one is based on combination of solvent partitioning (liquid–liquid extraction procedure with ethyl acetate) and strong anion exchange chromatography (QAE Sephadex A25) followed by $C_{18}$ solid phase extraction procedure. Samples are subsequently fractionated using preparative HPLC, each fraction is methylated with ethereal diazomethane and subjected to trimethylsilylation with either *N*-methyl-*N*-trimethylsilyltrifluoracetamide[42] or with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide[43] before final analysis of GAs as methyl ester trimethylsilyl ethers by gas chromatography-mass spectrometry (GC-MS). Alternatively, liquid–liquid extraction and HPLC can be replaced by solid phase extraction (SPE) methods. SPE-columns are packed with solid sorbent of different chemical properties and the analyte (GAs) is bound to the column by chemical interaction with the solid phase. Interfering substances are removed by washing the column with a suitable solvent and GAs are then eluted from the column using stronger solvent. Using a different separating mechanism in each of the purification steps improves the purification efficiency. SPE-based purification method using mixed-mode columns are suitable for use

with purification robots, which speeds up sample preparation allowing higher throughput in the analyses. A combination of different mixed-mode SPE columns for purification of GAs prior to analysis by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) allows their analysis as free acids without derivatisation[39].

### *3.2. Analytical Methods*

#### 3.2.1. Identification of Gibberellins

When analysing new plant material, qualitative analysis is necessary in order to confirm the presence of specific known GAs in the tissue of interest or to identify new GAs. Since identification of any organic compound including GAs requires a physicochemical detector that is able convincingly distinguish structurally similar compounds from each other, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most common techniques fulfilling this condition. Although NMR is an eminently useful tool for identification, it has low sensitivity compared with MS and is usually not suitable for analysing the extremely low concentration of GAs and other plant hormones present in most plant tissues. Moreover, physicochemical techniques cannot be readily used for analysis of complex mixtures of samples such as plant extracts without separation of the components. The best opportunity to identify small amounts of previously known compounds in a purified extracts is a combination of MS with an efficient chromatographic system such as capillary GC or ultra performance liquid chromatography (UPLC). The revolutionary development of MS in terms of construction of tandem instruments (triple quadrupole, ion trap, quadrupole time-of-flight and other hybrid instruments) has greatly improved the accuracy of identification data and simplified qualitative analysis. The improvement of the selectivity of the analysis in tandem MS is achieved by removing most of the potentionally interfering compounds by selecting the GA parent ion using the first mass analyser. The second one then detects ions formed by the fragmentation of the parent ion. Mass spectrometry therefore has become the analytical method of choice. Nevertheless, there are occasions when NMR must be used to obtain complete structural information, e.g. for previously uncharacterised GAs or determining sugar positions in GA conjugates etc.

As mentioned earlier, samples containing GAs can be introduced into the mass spectrometer ion source in either gas phase (GC-MS, in which

the mass spectrometer serves as a highly versatile GC detector)[44] or alternatively in liquid phase (LC-MS). The identity of GAs is confirmed by comparison of their Kovats retention index (KRI)[45] and the mass spectra obtained with those of pure standards or alternatively to published spectra[46]. Mass spectra for all identified GAs can be found at www.plant-hormones.info/ga1info.htm. GC analysis of GAs requires derivatisation to increase their volatility. The carboxylic acid groups of GAs are usually converted to methyl esters with ethereal diazomethane and the resulting methyl esters are less polar than the free acids. The sample may be analysed by GC at this stage but it is more common to convert hydroxy groups of GAs methyl esters to trimethylsilyl (TMS) ethers[38] (formation of MeTMS GAs) that further decreases the polarity of the emergent molecule and, more importantly, improves its mass spectral characteristics. The free acids can also be converted to TMS derivatives directly without previous methylation. The most common reagents for trimethylsilylation are BSTFA (*N*,*O*-bistrimethylsilyltrifluoroacetamide)[42,47] and MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide)[43,48], which is slightly more volatile. Both silylating reagents are highly reactive and more volatile than derivatised GAs of interest, so they are well separated from them on the GC column. Nevertheless, certain vicinal diols are incompletely silylated, as it is for the glucose moiety in GA-glucosides, and more specific derivatives can be prepared in order to confirm the structure (for example acetonides by reaction with acetone[49]). After derivatisation, GAs are separated on the GC column and introduced into the mass spectrometer, where they undergo extensive fragmentation. The mass spectra can be obtained by different ionisation techniques. Electron impact (EI) ionisation is mostly used for production of fragments from the MeTMS GA molecular ions[50,51]. Fragmentation is achieved by bombardment with high-energy electrons (usually 70 eV) under high vacuum resulting in the formation of both positive and negative ions, although it is more usual to measure positive EI spectra. Total ion current chromatograms (TIC) are generated by summing the ion currents from each scan and the resulting trace indicates the composition of the sample. Each mass spectrum serves as fingerprint for the individual compounds, which is then compared with published spectra or spectra from authentic standards. Chemical ionisation is an alternative to EI and it is used for GAs that give no molecular ions in EI [52]. Ionisation takes place in the presence of a large excess of a reagent gas (such as methane) which is ionised by EI and transfers its charge to the sample by collision via hydrogen or a heavier fragment. CI offers fewer fragments since the ionisation process involves less energy.

In practice, the identifications based on comparison of mass spectra, require a great deal of judgement. GC retention time is an additional parameter that considerably aids identification. When it is possible to compare both mass spectra and retention times, identification can be made with reasonable sureness. In the case of GA epimers such as $GA_{34}$ and $GA_{47}$ or $GA_{48}$ and $GA_{49}$, identical mass spectra are obtained, so these GAs can be distinguished only based on retention times when using GC-MS.

Liquid chromatography has been also used for qualitative analysis of derivatised GAs[50,53–55], but the selectivity of detection (UV or fluorescence) is not usually sufficient to allow unequivocal identification since all carboxylic acids are derivatised similarly.

Although there are currently over 130 GAs for which the structures are known, there are still uncharacterised GAs to be identified. Modern strategies of elucidating GA structure are determined by the amount of compound available. If sufficient sample is available, chemical characterisation of the compound can be attempted in terms of infrared, NMR and MS. However, since it is not usually practical to obtain sufficient GA from plants for full structural analysis, identification of new GAs is usually accomplished by chemical synthesis of the putative structure and comparison with the natural compound by MS.

### 3.2.2. Metabolic Studies

Transport, *de novo* synthesis, conjugation, catabolism and compartmentation are metabolic processes that help maintain the plant hormone content in plant cells at optimal levels for growth and development. For metabolism studies of GAs, methods involving isotopically labelled compounds as tracer are in use. The labels that can be used are the radioisotopes $^{3}H$ and $^{14}C$ (recently also $^{17}C$)[60], and the stable isotopes $^{2}H$, $^{13}C$, $^{18}O$ and $^{17}O$. Most investigators prefer the radioisotopes since they simplify the detection of products and the determination of recoveries and conversion efficiencies. The detection of metabolites labelled with stable isotopes is limited to MS and NMR. The choice of label depends on the application. It may be determined by the methods available for introducing the isotope into a particular substrate or by the amount of conversion expected. Tritiated compounds can be detected at much lower concentrations since they can be synthesised with specific radioactivities up to a thousand times higher than those labelled with $^{14}C$. Radioisotopes are detected after chromatographic separation on line after GC when effluent is directed to a FID

detector to provide a mass trace[61]. The FID effluent, [$^3$H]$H_2O$ or [$^{14}$C]$CO_2$, is then trapped, mixed with scintillation fluid and counted in a liquid scintillation counter[62] as an inexpensive method. Tritium-labelled GAs detected by GC-MS were used for feeding experiments to study their metabolism in immature seeds of *Pisum sativum*[63]. Using LC as the chromatographic system, radioactivity is monitored by a radiodetector[64] where the scintillation fluid is mixed with the LC effluent. Depending on the LC solvent, counting efficiencies ranged between 60 and 80% for $^{14}$C and 8–35% for $^3$H. Heterogeneous counting systems with solid scintillant are also available. They are non-destructive and simple to use, but obtained counting efficiencies are less than those achieved with the homogenous system.

### 3.2.3. Quantification of Gibberellins

Gibberellins can be analysed by GC-MS as methyl ester trimethylsilyl ether derivatives as mentioned above. Because of the large number of GAs present in a single plant tissue, it is necessary to use highly selective multiple reaction monitoring detection (MRM) to provide sufficient selectivity in quantitative analysis, which needs to overcome problems arising from the presence of isomeric GAs ($GA_1$ and $GA_{29}$), GAs co-eluting from the GC column and the occurrence of many interfering compounds[65,66]. The quantitative analysis of GAs is usually achieved by the isotope dilution method giving very precise determination as described by Croker et al.[67]. Liquid chromatography-mass spectrometry (LC-MS) is becoming more common for quantitative analysis of GAs. In 2007, Varbanova et al.[25] reported the method of analysis of GAs as free acids in Arabidopsis mutants by LC-MS/MS after a labour-intensive five-step purification of plant tissue extracts. Quantification was achieved using isotope dilution analysis based on [17,17-$^2$H]-GAs added before purification. UPLC was equipped with a reversed-phase $C_{18}$ column, the effluent from which was introduced into a quadrupole/time-of-flight tandem mass spectrometer. The authors separated 14 GAs within 16 min using a water-acetonitrile gradient. However, the detection limits for compounds of interest were not published. This method of extraction and analysis was successfully used also for determining the endogenous GA profile during Christmas rose (*Helleborus niger* L.) flower and fruit development[68].

UPLC-MS/MS was also used for the quantification of 11 GAs (as negatively charged compounds) by Kojima et al.[39] in 2009, who employed an improved purification procedure. These authors also described a method to enhance the sensitivity of the method: they esterified the carboxylic acid

groups with bromocholine, allowing the more sensitive positive ion detection. Quantification limits of the modified substances were enhanced from 50 to 1 fmol, in the case of $GA_1$.

### 3.2.4. Gibberellins in Hormone Profiling

Most plant scientists involved in plant hormone analysis usually investigate only one hormone class at a time. However, it is well known that hormones interact with each other and have mutual effects on their biosynthesis and catabolism. For this reason, there is an increasing interest from plant physiologists in analysing several hormones in the same sample. This can be achieved by dividing the sample into different parts and then analysing each part for a different hormone. However, this requires relatively large samples and is very time-consuming. Alternatively, several hormones can be analysed in the same sample; this requires a method that enables extraction, purification and detection of all the major hormones in the same extract. For example, during a study of thermodormancy, cytokinins, 3-indoleacetic acid, abscisic acid and GAs were all measured in the same extract of lettuce seeds by LC-MS operated in electrospray ionisation mode[40] (ESI). ESI is a soft ionisation technique and one of the most effective interfaces for liquid chromatography and capillary zone electrophoresis (CZE) allowing an ions, present in an effluent of a separation system, to be be transferred to the gas phase by applying a voltage at atmospheric pressure. The authors analysed all hormones in 40 min using only one-step of purification of the plant extract from 50–100 mg DW (dry weight) of material prior to the analysis. The UPLC-MS/MS was further used for simultaneous analysis of the same molecular species in rice GA-signalling mutants[39] as mentioned in the previous paragraph.

## 4. CONCLUSIONS

Research in the field of GAs, one group of plant chemical messengers, takes place at many levels – molecular biology, genetics, biochemistry, cell biology, plant physiology, etc. The results of investigations and observations coming from all of these disciplines have enabled the identification of the majority of the genes encoding enzymes in the GA metabolism pathway in model plant species, discovery of GA deactivation enzymes and the identification of some transcription factors that directly regulate GA metabolism genes. There is also compelling evidence that bioactive GAs act as key mediators in growth responses to environmental cues, such as temperature and

light. The findings of biologists have to be supported in many cases by quantitative analysis of GAs in plant tissue or any other biological material such as fungi, cell cultures etc. Their occurrence in plant tissues at extremely low concentrations (in general pg $g^{-1}$ of fresh weight) requires very sensitive analytical tools. With regard to the high complexity of matrix, the need for thorough purification providing high enrichment of these phytohormones is essential prior to the detection by standard analytical techniques. At the present time, GAs are analysed predominantly by chromatographic methods (liquid, gas chromatography) coupled to MS after tedious, labour intensive and time–consuming preconcentration steps comprising SPE using general purpose sorbents. The future issues in this field could thus be the development of highly selective, flexible and environmentally friendly solid-phase extraction sorbent for the high enrichment and efficient clean-up of GAs from complex plant extracts in a single step prior to their qualitative and quantitative analysis using hyphenated techniques.

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