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Family Business: Multiple Members of Major Phytohormone Classes Orchestrate Plant Stress Responses

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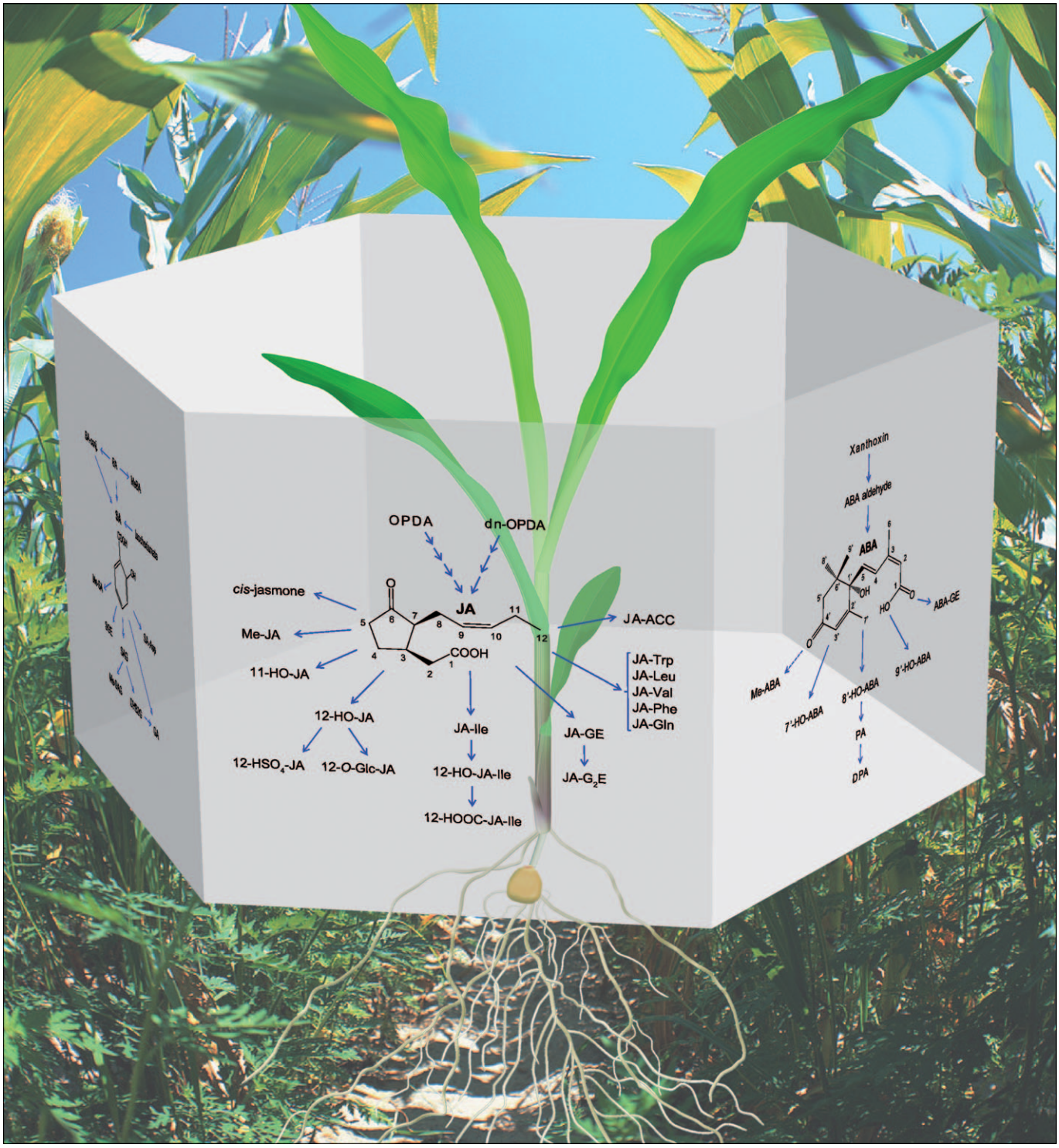

Abstract: Low-molecular-weight compounds such as jasmonic, abscisic and salicylic acids are commonly thought to be regulators of plant stress responses. However, it is becoming clear that these molecules, often referred to as phytohormones, are only a part of bigger groups of compounds with biological activity. We propose that the concept of "hormone families" may help to better understand plant physiological responses by taking into account not only the alleged main regulators, but also their precursors, conjugates and catabolites. Novel approaches to profile potentially active compounds in plants are discussed.

Keywords: analytical chemistry · analytical methods · natural products · phytohormones · plant behavior · structure–activity relationships

Introduction

Plants are very quick and efficient in adjusting their phenotype to a changing environment. Upon pathogen or insect attack, they start producing a variety of toxic secondary metabolites and proteins,[1–3] they increase the emission of volatile signalling compounds,[4] they change their morphology and growth patterns,[5,6] and they reallocate photo assimilates to non-attacked tissues.[7] Together, these adjustments considerably improve the plant's chance of surviving the attack and producing viable seeds.[8] Many of the defensive responses of plants are thought to be mediated by stress phytohormones, a group of structurally unrelated small molecules, including, among others, jasmonic (JA), salicylic (SA) and abscisic (ABA) acids. The idea that these compounds are major players in plant stress responses has been based on several observations: Firstly, all of these compounds increase in concentration after insect or pathogen attack,[3,9,10] often preceding other phenotypic adjustments.[11,12] Secondly, their exogenous application partially mimics the natural stress responses of plants.[13–15] Thirdly, mutants that are compromised in the biosynthesis or perception of these molecules become more susceptible to insect and/or pathogen attack.[16–18] Although these observations do not directly prove the biological activity of the above-mentioned molecules, they have led to the common view that a plant's defensive state can reliably be predicted by measuring single phytohormone levels,[19–21] and that research on plant–environment interactions should be based on models of phytohormone networks.[22,23] However, as chemists and biochemists are advancing our understanding of phytohormone signalling and perception, it is becoming increasingly clear that this view may be inaccurate: JA, for example, a compound that has long been thought to be the major driver of a plant's defensive response to insect attack, may not be that important after all. In *Arabidopsis thaliana*, the isoleucine conjugate JA–Ile has been shown to be much more active than JA itself,[24] leading researchers to think of JA as a prohormone.[25] Furthermore, whereas JA is restricted to plant cells and vascular tissues, its methylated form (MeJA) as well as *cis*-jasmone are volatile and can therefore easily reach distant plant parts and other organisms.[26,27] Finally, precursors, such as 12-oxophytodienonic acid (OPDA), that have formerly been thought of as simple intermediates necessary for JA synthesis are likely to be active themselves.[28] Thus, instead of seeing JA as a single phytohormone, it would be more appropriate to refer to it as one of many members of the phytohormone family of "jasmonates". While this notion is well accepted in the case of JA and other hormones, such as gibberellins, there is much less awareness about similar patterns that may be present for other stress phytohormones.

Herein, we discuss the family concept by compiling our current knowledge about the possible chemical and functional diversity of major stress phytohormone classes, including jasmonates (related to JA), abscisates (related to ABA) and salicylates (related to SA). Contrary to other classifications that are purely based on specific chemical reactions, our approach combines biological and chemical knowledge. Compounds are included into "phytohormone families" if they are 1) chemically related to the main compounds and 2) have some potential biological activity. We argue that by adopting the concept of hormone families, a more complete understanding of plant–environment interactions can be attained. For more detailed reviews on the biosynthesis and catabolism of plant hormones as well as their interplay upon environmental stress, we recommend the numerous recent articles that have been published on the subject.[22,29–32]

Jasmonates: Diversity and Function

(−)-Jasmonic acid (JA) was originally described as a plant growth regulator.[33,34] The discovery that JA partially mimics plant wound responses,[14] spurred great interest among biologists for this compound. Although the fact that JA is only one metabolite of a diverse family of compounds

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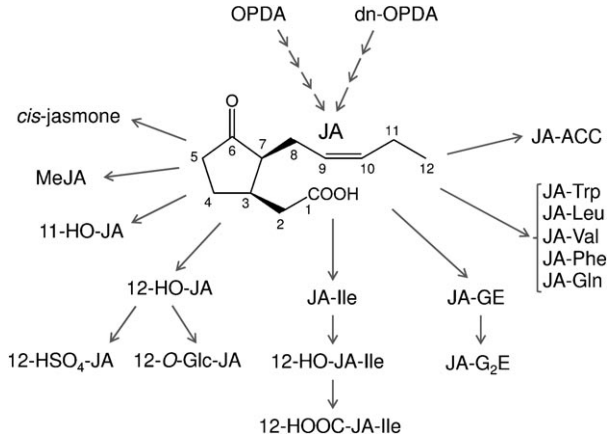
(Scheme 1) had long been recognised,[35] much of the subsequent research efforts were centred around this putative main driver of plant–insect interactions.[11,12,23,36] Recent studies, however, emphasise that JA by itself may neither be the best indicator nor the best mimic for the jasmonate-dependent reaction of the plant to stress.



Scheme 1. Plant regulators of the jasmonate family. OPDA: oxophytodienoic acid; dn-OPDA: dinoroxophytodienoic acid; JA: jasmonic acid; 11-HO-JA: 11-hydroxyjasmonate; 12-HO-JA: 12-hydroxyjasmonate; 12-HSO_4-JA:12-hydroxyjasmonate sulphate; 12-*O*-Glc-JA: 12-hydroxyjasmonoyl glucoside; JA–Ile: jasmonoyl isoleucine; 12-HO-JA–Ile: 12-hydroxyjasmonoyl isoleucine; 12-HOOC-JA–Ile: 12-carboxyjasmonoyl isoleucine; JA-GE: jasmonoyl-1-β-glucose ester; JA-G_2E: jasmonoyl-1-β-gentiobiose ester; JA-Trp: jasmonoyl tryptophan; JA-Leu: jasmonoyl leucine; JA-Val: jasmonoyl valine; JA-Phe: jasmonoyl phenylalanine; JA-Gln: jasmonoyl glutamine; JA-ACC: jasmonoyl 1-aminocyclopropane-1-carboxylic acid; Me-JA: methyl jasmonate.

The search for the jasmonate receptors has unveiled that in *Arabidopsis* two proteins, COI1 and JAZ, form a complex together with the isoleucine conjugate, JA–Ile, to induce jasmonate-dependent gene expression.[37] Since JA itself cannot promote this interaction, JA–Ile is now considered to be the main regulatory metabolite in COI1/JAZ downstream processes in *Arabidopsis*. Interestingly, (−)-JA–Ile, the major product of JA-derived JA–Ile isolated from plants, was shown to be much less active in plants than coronatine, a JA homologue derived from bacteria.[38] Following this, (+)-7-*iso*-JA–L-Ile was identified as the active form of the hormone.[39] The pH-dependent epimerisation in the cytosol may regulate the abundance of the active isomer,[38] thereby providing a mechanism to change hormone activity independently of the biosynthetic activity of the pathway.[31] Signal inactivation may also be accomplished by hydroxylation (12-HO-JA–Ile)[40] and carboxylation (12-HOOC-JA–Ile).[41]

In plants, JA can be conjugated with a series of other amino acids, including leucine (Leu), valine (Val), alanine (Ala), tryptophane (Trp), glutamine (Gln) and phenylalanine (Phe).[24,42–44] Whereas in *Arabidopsis* none of these conjugates seem to play an active role in COI1/JAZ signalling,[24,38] JA-Val and JA-Leu are reported to be active in tomato plants, in which JA–Val is as efficient as JA–Ile itself.[45] Furthermore, JA–Trp has been shown to inhibit auxin-dependent physiological responses in *Arabidopsis*.[43]

Reversible methylation of JA to methyl jasmonate (MeJA) has been described in numerous plant species. MeJA by itself does not seem to exhibit any biological activity in plants,[37,46] but has been implicated in volatile-mediated plant–environment interactions[47] and intra-plant signalling.[26] Another derivative of JA that is volatile is *cis* or (*Z*)-jasmone.[48] It has initially been proposed as a "disposal metabolite" that inactivates jasmonate signalling .[48] Later work, however, demonstrated that *cis*-jasmone is a highly active plant signal that repels aphids and attracts natural enemies.[27] It is furthermore becoming clear that *cis*-jasmone retains the potential to induce plant defences in *Arabidopsis*, some of which are even specifically responsive to this jasmonate.[49] Another noteworthy link for JA with volatile signalling has recently been indicated with the discovery of JA–ACC, a conjugate of JA and the precursor of ethylene (ET), 1-aminocyclopropane-1-carboxylic-acid (ACC):[24,44] conjugation to ACC might promote either JA inactivation or influence concentrations of free ET in the plant.[38]

Several studies also suggest that precursors of JA may be directly involved in COI1/JAZ-independent stress responses. OPDA regulates multiple wound-responsive genes and resistance in *Arabidopsis*.[28,50] Furthermore, applying exogenous JA–Ile to *Nicotiana attenuata* plants impaired in JA biosynthesis does not fully restore the plant's resistance to herbivores, providing further evidence for the notion that JA-upstream metabolites are important defence signals as well.[51]

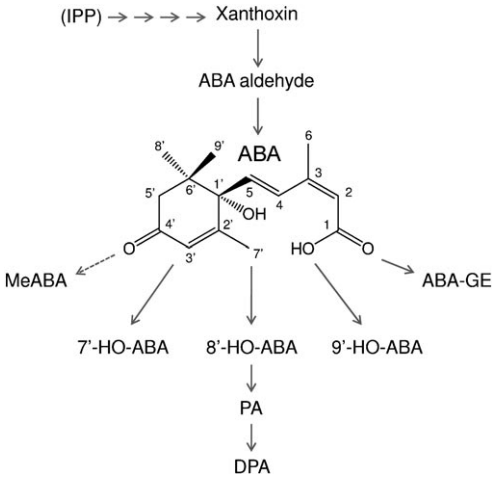
Hydroxylated forms of JA, namely, 11-HO-JA and 12-HO-JA, are abundant in many plant species, often in concentrations similar to or higher than JA.[52] 11-HO-JA was initially found in a plant in *Solanum demissum*[53] and is present in several other species.[52] The biological activity of 11-HO-JA remains largely unexplored, owing to the fact that it is likely to be formed non-enzymatically. Contrary to JA itself, 11-HO-JA seems to activate the glutathione S-transferase 1 (GST-1) gene, probably because of its labile hydroxy allylic group.[54] 12-HO-JA, initially described as a tuber-inducing compound in potato[55] accumulates systemically after wounding[41,56] and has shown to negatively regulate JA biosynthetic genes, suggesting that hydroxylation may contribute to a "switch-off" of JA signalling.[52] Similar effects have been described for its sulfonated form, 12-HSO_4-JA.[52] A specific enantiomer of the glucosylated derivative of 12-HO-JA, 12-*O*-Glc-JA, was found to induce nyctinastic leaf closure in *Albizzia* species.[57] Conjugation of a glucose moiety to JA can also occur at C-1, leading to jasmonoyl-1-β-glucose ester, jasmonoyl-1-β-gentiobiose ester, and hydroxyjasmonoyl-1-β-glucose ester.[58] Unlike JA, these molecules do not inhibit the G2 phase of the cell cycle in to-

bacco BY-2 cells.[58,59] So far, it remains unclear whether these glycosylated forms serve as JA storage or inactivation.

This non-exhaustive overview of the diversity and function of the jasmonates clearly demonstrates that the whole family rather than just JA contributes to plant stress responses and plant–environment interactions (Scheme 1). The available literature also suggests that plant species may differ in their jasmonate profiles and response patterns. Several other jasmonate-related compounds have yet to be tested for their activity, and new analytical approaches such as metabolomics may contribute to the discovery of yet unknown members of the family.

Abscisates

If JA itself only represents one member of a larger family of compounds, the question of whether other classical stress hormones follow a similar pattern seems pertinent. ABA, for example, is involved in plant responses to stress, including drought, osmotic stress, pathogen and insect attack,[30] and this diversity of function may stem from multiple active ABA-related compounds. Unlike JA, however, recent research suggests that ABA itself is indeed active. The latest evidence comes from studies elucidating the mechanism by which ABA binds to PYR/PYL/RCAR proteins, a class of receptors that have a prominent function in regulating SnR kinases involved in ABA downstream gene expression.[60–62] While the discovery of these (+)-ABA specific receptors may help to deepen our understanding of ABA signalling, this does not mean that other compounds in the ABA biosynthetic and catabolic pathways can be neglected (Scheme 2).



Scheme 2. Plant regulators of the abscisate family. ABA: abscisic acid; Me-ABA: methyl abscisate; 7′-HO-ABA: 7′-hydroxyabscisate; 8′-HO-ABA: 8′-hydroxyabscisate; 9′-HO-ABA: 9′-hydroxyabscisate; ABA-GE: abscisyl-1-β-glucose ester; PA: phaseic acid; DPA: dihydrophaseic acid; IPP: Isoprenoids.

It has been suggested that the ABA glucose ester (ABA-GE) rather than ABA itself may be the actual mobile signal.[63] This hypothesis is especially appealing because contrary to ABA, ABA-GE is highly hydrophilic and can therefore be translocated in the xylem of plants without any loss to the surrounding tissues.[63] Specific unloading and conversion could then occur in the target tissue.[63] Interestingly, ABA-GE was also identified as an active compound of *Citrus junos* plants that inhibited root growth of lettuce seedlings at concentrations of 1.4 μM.[64] It was furthermore suggested that ABA-GE may be involved in plant–pathogen interactions.[65] In this context, it should be noted that ABA-GE is only one of a series of ABA-conjugates that have been found in the xylem vessels of plants. In sunflower plants, at least five additional alkaline, hydrolysable ABA conjugates were discovered, one of which was only present in water-stressed plants.[66] Comparable patterns have also been reported for barley.[67] As yet, the precise role of ABA-GE and other conjugates remains to be elucidated.[68]

Apart from reversible conjugates, several true catabolites of ABA have been described.[69] The major breakdown products of ABA are commonly thought to be (−)-phaseic acid (PA) and (−)-dihydrophaseic acid (DPA).[30] There is little information available about their biological role apart from maintaining ABA homeostasis, but the precursor of PA, 8′-hydroxyabscisate (8′-HO-ABA) as well as two other hydroxylated forms clearly possess biological activity: 9′-hydroxyabscisate (9′-HO-ABA) exhibited ABA-like inhibition of *Arabidopsis* seed germination.[70] Furthermore, a recent study shows that 7′-hydroxyabscisate (7′-HO-ABA) and 9′-HO-ABA induce oleosin and fatty acid elongase genes as well as the accumulation of long chain fatty acids in *Brassica napus* embryos. In the case of 9′-HO-ABA, the reaction was even stronger than the one elicited by ABA itself.[71] 8′-HO-ABA treatment also increased oil synthesis, but had a weaker effect on gene expression.[71] Thus, the hydroxylated forms of ABA can thus by no means be regarded as simple breakdown products, but should be seen as active members of the abscisate hormone family. Mutants that are defective in ABA hydroxylases may help further establish the precise role of hydroxylated abscisates.

Methylated, volatile forms of phytohormones have been reported for a number of families, including the jasmonates and the salicylates (see below). Methylated ABA (MeABA) had originally been found in tomato shoots, but was later suspected to be an artefact of methanol extraction.[72] Although one study reported the natural occurrence of MeABA in stigma extracts of tobacco almost a decade later,[73] it remains unclear if this abscisate is of importance for plant stress responses. Yet, another class of volatile compounds that is intimately linked with ABA—the isoprenoids—has received attention lately. The latter are formed via the chloroplastic methylerythritol phosphate (MEP) pathway. The MEP pathway also yields the carotenoids that act as precursors of ABA.[74] It is therefore not surprising that in *Phragmites australis*, a close link could be observed between isoprenoid emission and ABA production.[75] Iso-

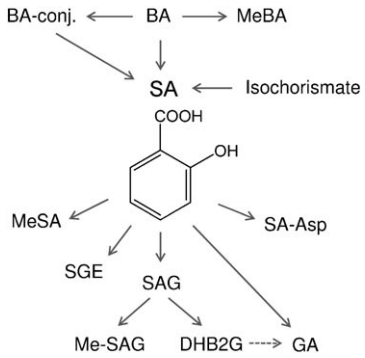
prenoids can thus convey specific information about the activation of abscisates in a plant to its environment. Given their increasingly recognised role in plant–environment interactions,[76] isoprenoids should be taken into account as potential "abscisate messengers".

More immediate precursors of ABA should also be considered as potentially active abscisates. *cis,trans*-Xanthoxin, a carotenoid that is thought to be the primary precursor of MEP-produced ABA,[30] has been shown to be an active allelopathic agent of *Pueraria thunbergiana*.[77] Further evidence for its biological activity comes from early studies showing inhibitory effects on wheat coleoptiles, lettuce hypocotyls and bean petioles.[78] In the same study, the effect of *cis,trans*-xanthoxin on cress seed germination was even significantly more pronounced than that of ABA itself,[78] suggesting that it is not simple precursor conversion that accounts for the activity. ABA is synthesised from xanthoxin via abscisic aldehyde, which seems to exert some biological activity as well. It has been implicated in both stomatal closure[79] and allelopathy.[78]

Overall, evidence points to ABA being only one metabolite of a large family of highly bioactive compounds (Scheme 2). Instead of talking about ABA precursors, conjugates and catabolites, it may be appropriate to refer to the ensemble as "abscisates". Very little attention has been paid to many abscisates regarding their potential role in plant–insect and plant–pathogen interactions. Given the fact that the role of ABA itself in these processes is still under debate,[3,17,80] profiling the whole family of abscisates for their reaction upon attack may prove more than worthwhile.

Salicylates

SA is another classical phytohormone that has been implicated in the immune reaction of plants. SA is active against a wide range of attackers, including viral, microbial and fungal pathogens.[32] As in the case of JA and ABA, a number of important conjugates have been described (Scheme 3). Methyl salicylate (MeSA) is directly produced from SA in plants and subsequently released as a volatile compound. Although biologically inactive by itself, MeSA has been proposed to function as a non-vascular signal in tobacco plants, inducing resistance in non-attacked plant parts.[81,82] Recent research in *Arabidopsis*, however, has shown that most MeSA is released into the atmosphere rather than being retained by the plant, and that, at least in this particular system, it is not required for systemic resistance.[83] MeSA formation and release may therefore function as a catabolic process, reducing the amount of active SA in plant cells. The possibility that this mechanism can be "highjacked" by pathogens to lower the plant's defensive capacity has been suggested.[83,84] Interestingly, it is also becoming evident that MeSA may serve as an environmental cue: predatory mites are attracted to plants that emit MeSA after feeding by spider mites, their potential prey.[85,86]



Scheme 3. Plant regulators of the salicylate family. BA: benzoic acid; Me-BA: methyl benzoate; BA-conj.: benzoate conjugates; MeSA: methyl salicylate; SGE: salicyloyl glucose ester; SAG: salicyloyl glucoside; Me-SAG: methyl salicyloyl glucoside; DHB2G: 2,5-dihydroxybenzoic acid 2-β-glucose; GA: gentisic acid; SA-Asp: salicyloyl aspartate.

As found for JA and ABA, non-volatile SA conjugates include several glucosylated forms: salicyloyl glucose ester (SGE) and salicyloyl glucoside (SAG) in particular have been described.[87] While the function of SGE remains unknown, SAG is pathogen inducible and can be transported from the cytosol into the vacuole, thereby possibly serving as an SA storage form.[88] MeSA can also be glucosylated, yielding Me-SAG.[32] The function of Me-SAG remains unexplored. Interestingly, a hydroxylated form of SA (2,5-dihydrobenzoic acid, also called gentisic acid, GA) has been shown to be able to induce the production of pathogenesis-related (PR) proteins in tomato plants that are not inducible by SA itself.[89] GA has also been shown to accumulate in other plants after virus attack and to induce peroxidase activity similar to SA.[90] GA may also be formed via the SA-derived, abundant 2,5-dihydroxybenzoic acid 2-*O*-β-D-glucose (DHB2G).[87] Hydroxylated forms of SA, similar to what is reported for ABA, should be regarded as potentially active salicylates. This may be especially relevant for monocot plant systems that seem to have constitutively high levels of SA and that show resistance responses that are SA independent.[91–93]

While in tobacco plants and *Arabidopsis* most of the SA is produced via isochorismate,[32,94] it has been suggested that at least some SA is derived from benzoic acid (BA).[29] BA is induced after attack,[95] induces pathogen resistance when applied to *Banksia attenuata*[96] and has long been known for its general antifungal activity.[97] BA may thus serve both as SA precursor and active metabolite in plants. Interestingly, labelling studies in tobacco suggest that SA may be produced from BA via BA conjugates that accumulate rapidly after viral infection.[98] The nature of these conjugates as well as their actual importance remains to be demonstrated.[99] Given the diverse roles of benzoates in the metabolism of plants,[100] it can be anticipated that further links with salicylates may prove important in plant–hormone signalling.

The conjugation of SA with the amino acid aspartic acid (Asp) has been shown to occur in a number of plant species, including grapevine, common bean and *Arabidopsis*.[101] Although transgenic plants with higher levels of SA-Asp also

show an increased response of PR-1 to pathogen infection, the precise function of the amino acid conjugate remains unclear.[101] As is the case for ABA, the current literature provides multiple examples of potentially important salicylates in plant stress responses (Scheme 3). Profiling the whole family rather than only SA itself is certainly warranted to provide a more complete picture about phytohormone changes after stress onset. Applying novel analytical techniques may also lead to the discovery of as yet unknown SA-related compounds that are of potential biological relevance.

Analytical Approaches to Profile Phytohormone Families

Phytohormones are often present in trace amounts in plants. For example, levels of JA in unstressed *Arabidopsis* specimens are estimated to be approximately 20 pmol g^{-1} of the fresh weight (FW).[102] Amounts of ABA after herbivore attack do not exceed 20 pmol g^{-1} FW in maize shoots and 70 pmol g^{-1} FW in maize roots.[3] Consequently sensitive analytical protocols are needed for their detection, identification and quantification. In this section, we present an insight into the techniques that have been used for phytohormone analysis and we evaluate their ability to simultaneously profile various families of phytohormones, including known and possibly new derivatives.

Traditionally, targeted and selective approaches have been employed for the monitoring of plant hormones. A typical example is the use of radio immunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA).[103–105] However the repeatability of such methods can be impaired by the low purity of the extracts, unless extensive purification protocols such as preparative liquid chromatography are used. Moreover, their extreme selectivity is clearly not compatible with a global profiling approach for all plant hormones. As an alternative, a large variety of analytical methods have been developed, taking advantage of the progressive advances in analytical technologies. The most common approach involves a chromatographic step with online selective detection, in which comparison of retention times with available standards is made. For instance gas chromatography electron capture detection (GC-ECD) was employed for the sensitive detection of ABA.[106] A fluorescence detector was coupled with liquid chromatography (LC) for detecting ABA and JA after derivatisation.[107] Again, only a few selected metabolites can be detected when using such approaches, which are thus not ideal for broader hormone profiling.

To overcome this limitation, mass spectrometry (MS) has been used for phytohormone analysis, since it often permits their unambiguous identification even in complex plant extracts. Moreover, due to its high sensitivity, selectivity and good linearity of response, MS coupled to chromatography allows for reliable quantification of chemicals present at minute levels. Therefore, it is not surprising that GC-MS and LC-MS have become the methods of choice for the analysis of plant hormones. GC-MS presents the advantage to yield very reproducible fragmentation patterns, which is useful for metabolite identification. However, only volatile and thermostable molecules can be directly monitored by GC-MS. Non-volatile compounds of molecular weight lower than 400–500 Da can be analysed after appropriate chemical derivatisation. The latter represents a tedious procedure, but is a mandatory step for most plant hormones. Chemical derivatisation may be followed, for example, by the simple purification protocol called vapour phase extraction (VPE), in which hormone derivatives are volatilised at elevated temperature and trapped on Super-Q adsorbent, then eluted with an organic solvent and injected into the GC system.[108] This permits the exclusion of all non-volatile substances that may interfere with the analysis. For global plant hormone profiling, GC-MS represents an interesting approach, although derivatives of molecular weight higher than 400 Da are difficult to detect. This is, for example, the case for glucosylated derivatives of JA and ABA. To detect these compounds, together with smaller molecules, the most promising approach is certainly LC-MS. The technique has rapidly evolved in the last 20 years thanks to the development of atmospheric pressure ionisation (API) sources such as electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI). LC-MS has several advantages for the analysis of plant extracts: firstly, compared with GC-MS, the sample preparation can be kept to a minimum. For most plant hormones, a simple extraction in a mildly polar solvent such as methanol or a mixture of methanol/water is sufficient. The resulting extract can then be directly injected into the LC system without further purification. This greatly reduces the time spent for sample preparation. Furthermore, molecules presenting very different physicochemical properties can be analysed in the same analysis. For example, JA and arabidopsides are easily monitored in a single run.[102,109] A drawback of LC-MS is that API sources produce little or no fragmentation. The identification of phytohormones has thus to rely on a single ion of the molecular species and its retention time, unless in-source fragmentation or tandem mass spectrometry (MS/MS) are employed to fragment the molecules. MS/MS used in the multiple reaction monitoring (MRM) mode allows for precursor-to-product transitions, which are habitually specific for particular compounds in the plant extract. Such configuration also increases the sensitivity of the mass spectrometer by decreasing the background noise, which is crucial for the quantification of trace metabolites. Triple quadrupoles and hybrid quadrupole-linear ion-trap systems are the most sensitive and thus most frequently used instruments for quantitative MS/MS. Wilbert et al. introduced LC-MS/MS for plant hormone quantification[110] and since then the technique has been increasingly used.[111–113] Recently, up to 17 molecules belonging to 7 different classes of phytohormones, including jasmonates, abscisates and salicylates have been simultaneously quantified by Pan et al.[114] For this, 14 closely related internal standards were added prior to tissue extraction. Thanks to

the very short dwell time of modern mass spectrometers and the possibility to create time windows within the analysis, the number of transitions that can be monitored in a single run is only limited by prior knowledge about the structure of the compounds of interest. Another important parameter is the availability of stable isotope internal standards (IS) for each dosed metabolite, which are essential for accurate quantification. Adding isotopically labelled IS prior to extraction provides correction for losses during sample preparation and for variable ionisation efficiencies.

When searching for new hormone derivatives, however, the MRM mode cannot be employed anymore. In this case, different approaches can be used and herein we present three different MS-based strategies for hormone discovery. The first one relies on the search for putative derivatives on the basis of a preliminary knowledge of biosynthetic and catabolic pathways, either in full scan or in selected ion monitoring (SIM) mode. For example, hydroxylated derivatives can be assumed to have a molecular weight that is 15.9949 Da higher than the original phytohormone. Similarly, the mass difference for a glucosylated derivative will be of 162.0528 Da relative to a given hormone. The use of a mass spectrometer enabling accurate mass measurements rather than a "nominal mass" instrument is obviously beneficial when investigating these mass differences. Currently, three analysers with this feature are commonly employed, namely, time-of-flight (TOF), electrostatic ion trap or Orbitrap, and Fourier-transform ion cyclotron resonance (FT-MS). Once a potential hormone derivative has been discovered, MS/MS can be used to generate fragments and confirm its identity. An alternative is to directly implement data-dependant MS/MS experiments. For example, ion trap and quadrupole TOF systems can easily be programmed to specifically fragment ions that meet some defined criteria. To assist the study of metabolism pathways, the exogenous application of stable isotopes of the original hormones can be performed and plants subsequently analysed.

The second approach utilises the precursor ion scan mode of tandem instruments such as triple quadrupoles in which the first and third quadrupoles function as mass analysers, while the second one serves as the collision cell. The experiment consists of selecting the fragment ion corresponding to a known hormone in the third quadrupole and scanning in the first quadrupole. Glucosylated derivatives are likely to be found by this means, because fragmentation releases the original hormone. As an example, the glucose and gentiobiose derivatives of JA with $[M-H]^-$ ions at m/z 371.1706 and m/z 533.2234, respectively, will fragment into a product ion at m/z 209.1178, which is typical of JA. However, this approach cannot be used for amino acid conjugates, for example, JA–Ile. Using MS/MS, the $[M-H]^-$ ion of JA–Ile at m/z 322.2018 will generate a fragment at m/z 130.0868 corresponding to the isoleucine moiety, with a neutral loss equivalent to a dehydrated JA molecule (192.1150 Da). A variant to precursor ion scan is precursor ion discovery (PID), which is most efficient with quadrupole TOF systems. In this configuration, the quadrupole is operated in RF-only mode and the instrument switches alternatively between low and high collision energy in the collision cell. At low energy, ions of the molecular species are formed, while at high energy fragments are generated. When a fragment ion of interest is detected, the instrument switches to the MS/MS mode and sequentially fragments each ion detected at low energy to reveal the true precursor. Compared with a conventional precursor ion scan, the duty cycle in the first quadrupole is increased by the elimination of scanning.

Finally, the third approach involves MS-based metabolomics, which aims at detecting and identifying all metabolites in a given organism.[115] Even though this represents a huge challenge, metabolomics has proven to be an efficient tool for the comprehensive profiling of plant samples.[116–118] The idea is to cover the widest possible range of metabolites to increase the chances to detect new phytohormones. To achieve this, various extraction methods, chromatographic techniques and/or stationary-phase chemistries, and ionisation modes may be used. The obtained data should then be treated by means of statistical data treatment tools to reveal phytohormones of interest among the high number of detected metabolites. To illustrate this kind of approach, the example of a plant stress response study is briefly presented:[119] the model plant *Arabidopsis thaliana* was submitted to wounding and metabolomics was employed to find original wound markers. Unwounded ($n=8$) and wounded ($n=8$) plants harvested 3 h after wounding were extracted and analysed by ultra-high pressure LC coupled to TOF-MS operated over a broad mass range (100–1000 Da) in both negative and positive modes. The data, which consisted in hundreds of features (m/z ions at a given retention time; Figure 1), were treated by multivariate analysis methods.

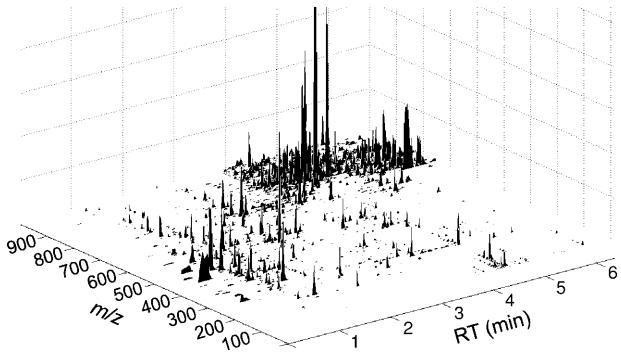


Figure 1. UHPLC-TOF 3D map of a wounded *Arabidopsis thaliana* extract.

In particular, principal component analysis (PCA), an unsupervised method, was found to be efficient to discriminate unwounded and wounded samples (Figure 2). Table 1 presents the loadings plot of the PCA shown in Figure 2, performed on data obtained in negative ion mode. It corresponds to a list of the features that contribute the most to

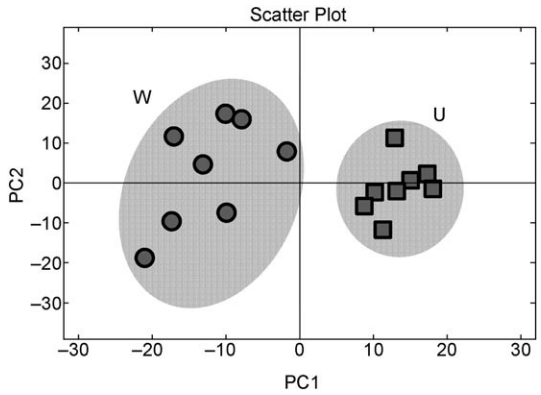


Figure 2. Principal component analysis score plot of unwounded (U, squares) and wounded (W, circles) specimens of *Arabidopsis thaliana*.

Table 1. List of the compounds contributing most to the discrimination between unwounded and wounded plants in the principal component analysis (Figure 2). The methods that were used for their identification are mentioned.[a]

PC1 score	RT [min]	*m/z*	Assignment
0.0677	2.3991	209.1172	JA: HRMS + std
0.0674	1.9405	417.1741	JA-Glc (formate adduct): HRMS + MS/MS
0.0673	4.2479	999.5155	?
0.0671	2.4009	277.1056	JA (sodium formate adduct)
0.0667	2.0504	352.1751	HOOC-JA–Ile: HRMS + MS/MS + NMR
0.0661	4.8951	926.4725	?
0.0661	2.4025	435.1723	OPC-4-Glc (Cl^- adduct): HRMS + MS/MS + NMR
0.0659	4.297	881.4846	?
0.0658	2.3964	445.2005	OPC-4-Glc (formate adduct)
0.0658	2.9811	519.2292	galactolipid?: HRMS + MS/MS
0.0648	3.0402	322.2017	JA–Ile: HRMS + MS/MS + std
0.0645	2.9874	488.2383	?
0.0644	1.343	225.1125	HO-JA: HRMS + NMR
0.0644	5.0483	397.2601	?
0.0643	2.642	327.2137	trihydroxylated fatty acid: HRMS + NMR
0.0638	2.9441	237.1496	OPC-4: HRMS + std
0.0637	2.0511	338.1941	HO-JA–Ile: HRMS + MS/MS + NMR

[a] RT: retention time, HRMS: high-resolution mass spectrometry, NMR: nuclear magnetic resonance spectroscopy, std: comparison with standard.

the discrimination between unwounded and wounded plants. Among them, several known jasmonates were identified on the basis of their molecular formulae and additional MS/MS experiments, as well as comparison with available standards. Other induced metabolites were unknown and further MS/MS and micro-NMR spectroscopy experiments were conducted to elucidate their structure.[41] By using this approach, a carboxylated derivative of the active hormone JA–Ile was discovered.[120]

From an overall point of view, the benefit of this method is that any wound-induced metabolite is likely to be detected, provided that it is ionised in the MS source. In other words, no preliminary hypothesis on the potential hormone derivatives had to be formulated. In conclusion, metabolomics can certainly be considered as the most exhaustive and hypothesis-free approach for plant hormone profiling.

Summary and Outlook

As research on the biosynthesis and activity of plant hormones progresses, it is becoming increasingly clear that a diversity of metabolites related to the different "classical phytohormones" play a role in plant defence and plant–environment interactions. Amino acid conjugation, hydroxylation, methylation and glucosylation, in particular, are prominent biochemical processes that diversify the metabolic arsenal of phytohormone families. The temporal and spatial patterns of these compounds within plant tissues may represent an additional layer of regulation that should be taken into account. New analytical approaches and a close collaboration between biologists and natural product chemists will help broaden our view on phytohormones and are likely to lead to the discovery of new regulatory elements of plant stress responses.

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