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Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography–electrospray tandem mass spectrometry

Xiangqing Pan [a,b,1], Ruth Welti [c], Xuemin Wang [a,b,*]

[a] Department of Biology, University of Missouri, St. Louis, MO 63121, United States
[b] Donald Danforth Plant Science Center, St. Louis, MO 63132, United States
[c] Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, KS 66506, United States

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Abstract

A rapid and sensitive method was developed for simultaneous quantification of multiple classes of phytohormones and some related metabolites in crude plant extracts without purification or derivatization. High-performance liquid chromatography and electrospray ionization–tandem mass spectrometry with multiple reaction monitoring were used to quantify auxins, cytokinins, abscisic acid, gibberellins, jasmonates, salicylates, and a number of related metabolites in crude plant extracts. The technology was applied to analyze biotic and abiotic stress-induced changes of phytohormones in Arabidopsis tissues, starting with 50–100 mg fresh tissue. Biotic and/or abiotic stresses were shown to differentially affect levels of salicylic acid, jasmonic acid, indole-3-acetic acid, and benzoic acid, in comparison to their methyl esters. Compared with previous methods, sample preparation time and amount of sample required for analysis of phytohormones are reduced, and more classes of hormones are quantitatively profiled. Structurally diverse compounds from complicated biological matrices are determined with high selectivity and sensitivity.

Keywords: *Arabidopsis thaliana*; Cruciferae; ESI–MS/MS; Hybrid triple quadrupole/linear ion trap mass spectrometer; Plant hormones; Metabolic profiling; Wounding; *Botrytis cinerea*

1. Introduction

Plant hormones are structurally diverse compounds that regulate plant growth, development, and response to biotic and abiotic cues. Phytohormones are grouped into several major classes including auxins, cytokinins, abscisic acid (ABA), gibberellins (GA), ethylene, jasmonates, salicylic acid (SA), and brassinosteroids (Davies, 1995, 2004; Crozier et al., 2000). While each class of hormones has characteristic biological effects, multiple plant hormones often mediate development and stress responses by additive, synergistic, or antagonistic actions. Crosstalk among auxin–cytokinin–ethylene in organ development (Aloni et al., 2006) and between ABA–GA (Gazzarina and McCourt, 2001), jasmonates–SA–ethylene (Reymond and Farmer, 1998), and ABA–jasmonates (Ho et al., 2003) in plant response to abiotic and biotic cues have been reported (Nemhauser et al., 2006). Availability of a simple and sensitive method to simultaneously quantify multiple classes of phytohormones will greatly facilitate investigation of hormone networks and functions (Reymond and Farmer, 1998; Gazzarina and McCourt, 2001; Ho et al., 2003; Aloni et al., 2006).

Extensive studies have been carried out to determine hormone levels in plants. Most work has been focused on analysis of a single compound, a single class, or a few classes of

* Corresponding author. Address: Department of Biology, University of Missouri, St. Louis, MO 63121, United States. Tel.: +1 314 516 6219; fax: +1 314 587 1519.
E-mail address: swang@danforthcenter.org (X. Wang).
[1] Solae Global Headquarters, 4300 Duncan Avenue, St. Louis, MO 63100, USA.

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hormones (Weber et al., 1997; Wilbert et al., 1998; Kowalczyk and Sandberg, 2001; Gomez-Cadenas et al., 2002; Chiwocha et al., 2003; Engelberth et al., 2003; Schmelz et al., 2003; Ross et al., 2004; Zhou et al., 2003; Durgbanshi et al., 2005; Lopez-Carbonell and Jauegui, 2005; Matsuda et al., 2005). Additionally, while it is becoming increasingly evident that hormone methyl esters play important roles in plant development and stress responses (Seo et al., 2001; Qin et al., 2005; Zhu and Park, 2005) and that formation of methyl esters may provide a distinctive way to regulate the activities of hormonal metabolism and function, measurements of naturally occurring hormone methyl esters in plant tissues have been scarce (Wilbert et al, 1998; Zhu and Park, 2005). Gas chromatography (GC) coupled with mass spectrometry (MS) is a powerful tool for hormone analysis due to its sensitivity (Weber et al., 1997; Kowalczyk and Sandberg, 2001; Engelberth et al., 2003; Schmelz et al., 2003). However, because GC analysis requires derivatization to enhance volatility and sensitivity, particular care must be taken in design of purification and derivatization methods if one is to distinguish methyl esters from free acids (Müller et al., 2002; Birkemeyer et al, 2003). Another potential downside in GC–MS procedures is that the required purification and derivatization procedures may involve considerable labor (Christie, 2003).

Liquid chromatography–electrospray tandem mass spectrometry (LC–ESI–MS/MS) has emerged as an effective method for phytohormone analysis. This approach has been applied to quantify individual classes of phytohormones, including ABA (Gomez-Cadenas et al., 2002; Ross et al., 2004; Zhou et al., 2003; Lopez-Carbonell and Jauegui, 2005), IAA (Matsuda et al., 2005), JA and SA (Durgbanshi et al., 2005). Also IAA, ABA and JA have been simultaneously determined from plant samples (Durgbanshi et al, 2005), and another recent strategy included auxins, cytokinins, ABA, and GA (Chiwocha et al, 2003). However, relatively large amounts of sample were used in these analyses, the target analytes were in most cases quite limited, and methyl esters were largely ignored. We describe here a simple and highly sensitive LC–ESI–MS/MS method that simultaneously quantifies molecular species from seven major classes of plant hormones including auxins, cytokinins, ABA, gibberellins, jasmonates, salicylates, and relevant methyl esters (see Fig. 1 for the structures) from crude plant extracts.

2. Results and discussion

2.1. Optimization of precursor-to-product ion transitions

SA, benzoic acid (BA), IAA, indole-3-butanoic acid (IBA), indole-3-carboxylic acid (ICA), ABA, cinnamic acid (CA), gibberellins (GA_3 and GA_4), JA, 13-*epi*-12-oxo-phytodienoic acid (OPDA) (Fig. 1), and their internal standards were analyzed in negative scan mode as $[M-H]^-$ ions; while salicylic acid methyl ester (MeSA), MeIAA, cinnamic acid methyl ester (MeCA), MeJA, benzoic acid methyl ester (MeBA), zeatin (Fig. 1), and their internal standards were analyzed in positive mode as $[M+H]^+$ ions, using ESI–MS/MS. Precursor and product ions specific for each hormone were identified, using authentic compounds (Fig. 1) and appropriate precursor-to-product ion transitions representing a major fragmentation path and unique for each phytohormone were chosen (Table 1). MS/MS conditions were optimized to produce maximal signal (Table 1).

2.2. Separation and identification by LC–ESI–MS/MS

To separate individual phytohormones in a mixture, each of the analytes and internal standards were subjected to chromatography on a C18 reversed-phase column, followed by analysis via ESI–MS/MS. Eluates were monitored by a series of multiple reaction monitoring (MRM) scans with one time of scan mode change using 700 ms as settle time, with 20 ms dwell time for each precursor-to-product transition, and a pause between mass ranges of 5 ms. Typical LC–ESI–MS/MS chromatograms of 17 phytohormones, related metabolites, and internal standards are shown in Fig. 2. Extracted ion current (XIC) from the positive and negative scan modes of hormones (Fig. 2B and C) can be extracted from total ion current (TIC) that combines positive and negative scans (Fig. 2A). Although some compounds co-eluted, such as IAA and BA, their different precursor-to-product ion (MRM) transitions allowed specific detection of each compound (Fig. 2D and E). This was also the case for CA and SA (Fig. 2F and G).

Applying the same approach to analyzing crude plant extracts with internal standards added during extraction yielded the chromatograms shown in Fig. 2H, I, and J. In plant samples, monitoring one precursor-to-product ion transition typically resulted in the identification of several peaks including one at the retention time of the target phytohormone (Fig. 2H, I and J for JA, ABA and SA). These data indicate that a combination of the LC retention time and the diagnostic precursor ion-to-product ion transition are required, and together, they provide the specificity necessary for the quantification of these phytohormones in plant samples.

2.3. Quantification

The peak area of the diagnostic product ion (Table 1) under optimized conditions was used for quantification. A variety of synthetic, isotopically labeled, or modified compounds was selected as internal standards (Table 1), and these compounds were confirmed not to occur in Arabidopsis samples. The amounts (mol) of phytohormones were determined by comparison of the response to the internal standards (mol) added during extraction to the plant sample components. Addition of internal standards during extraction provides correction for hormone loss

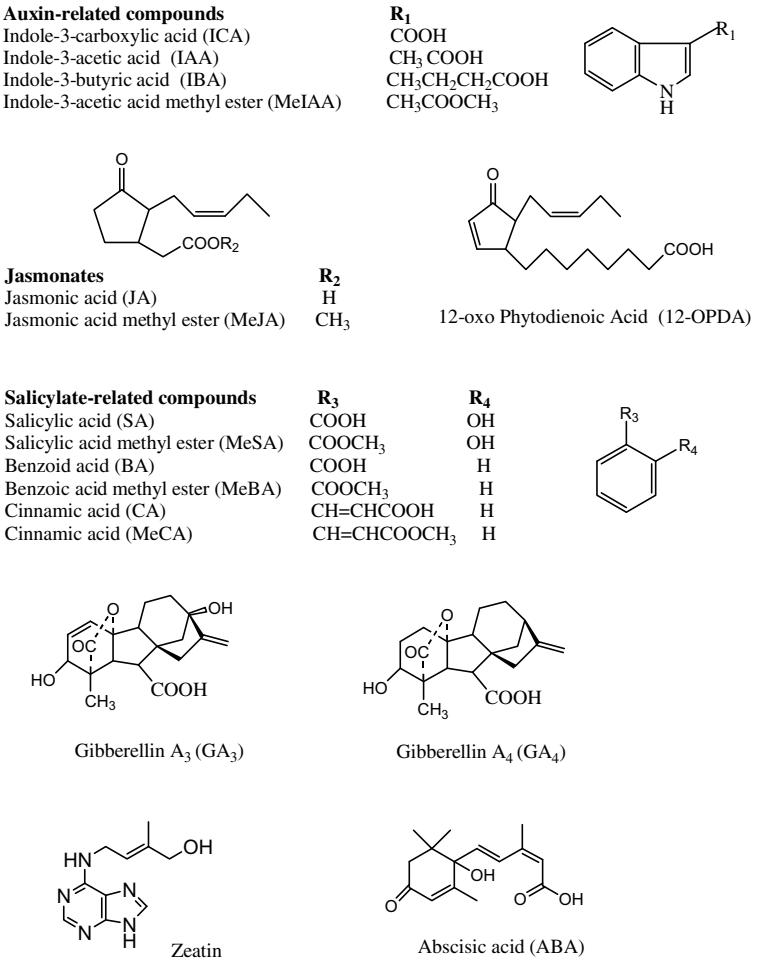

Fig. 1. Schematic structures of the phytohormones and related compounds studied.

Table 1
Optimized MS/MS conditions for the analysis of plant hormones

Analytes	Scan mode	Retention time (min)	Transition	Collision energy (eV)	Internal standard	Scan mode	Retention time (min)	Transition	Collision energy (eV)
Zeatin	+	2.91	220 → 136	29	d_5-zeatin	+	2.92	225 → 136	29
GA_3	−	4.29	345 → 143	−40	d_2-GA_3	−	4.31	347 → 143	−40
ICA	−	4.47	160 → 116	−22	d_5-IAA	−	5.05	179 → 135	−14
IAA	−	5.02	174 → 130	−14	d_5-IAA	−	5.05	179 → 135	−14
BA	−	5.9	121 → 76.8	−18	d_5-BA	−	5.99	126 → 82	−18
ABA	−	7.96	263 → 153	−16	d_6-ABA	−	7.98	269 → 159	−16
MeIAA	+	8.52	190 → 130	17	d_5-MeIAA	+	8.56	195 → 135	17
SA	−	9.45	137 → 93	−24	d_6-SA	−	9.48	142 → 98	−24
CA	−	9.8	147 → 103	−16	d_7-CA	−	9.88	154 → 110	−16
MeBA	+	9.94	137 → 105	11	d_5-MeBA	+	9.98	142 → 110	11
IBA	−	10.17	202 → 158	−16	d_5-IAA	−	5.05	179 → 135	−14
JA	−	10.44	209 → 59	−24	H_2JA	−	11.94	211 → 59	−24
MeSA	+	11.48	153 → 121	21	d_4-MeSA	+	11.49	157 → 122	21
MeCA	+	12.15	163 → 131	17	d_7-MeCA	+	12.18	170 → 137	17
MeJA	+	12.29	225 → 151	25	MeH_2JA	+	13.95	227 → 153	25
GA_4	−	13.11	331 → 213	−24	d_2-GA_4	−	13.12	333 → 215	−24
OPDA	−	19.38	291 → 165	−30	H_2JA	−	11.94	211 → 59	−24

during sample preparation and chromatography. Again, although some of the chromatographic peaks for analyte/internal standard pair overlapped, monitoring of distinct precursor-to-product ion transitions allowed determination of the internal standard and analyte (Fig. 2). The inclusion of internal standards, which elute in most cases simultaneously with the measured compounds, reduces any quantification problems that might be caused by potential

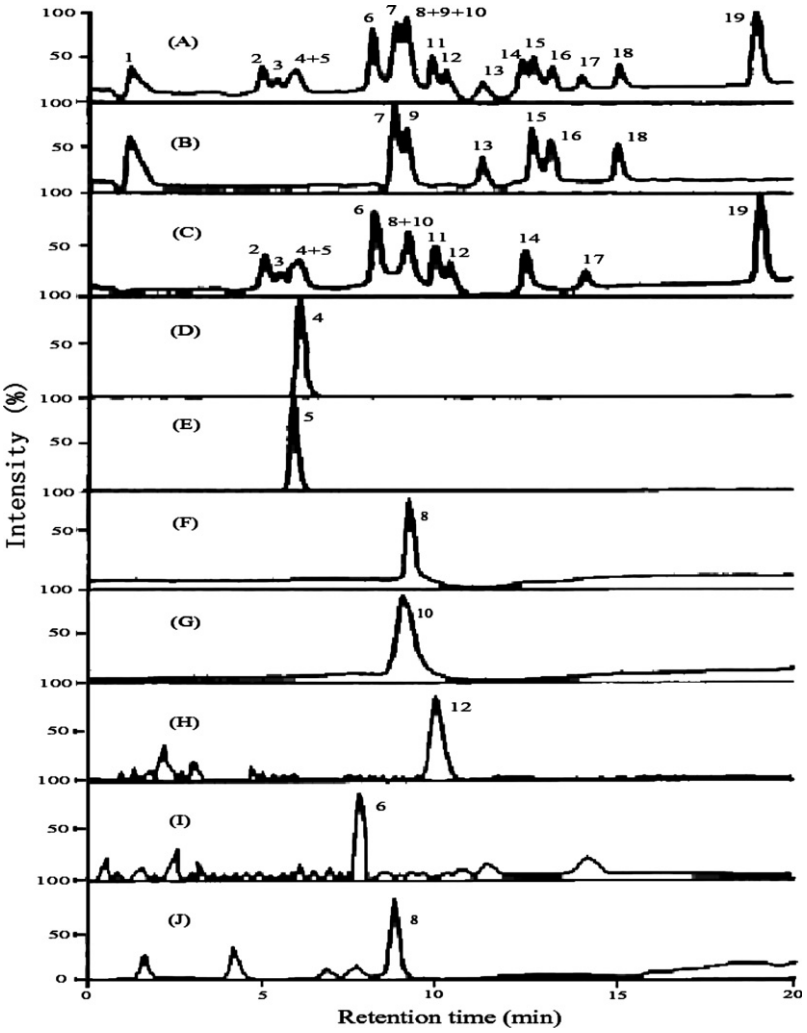

Fig. 2. Representative LC chromatograms of 17 plant hormones and their internal standards. (A) Total ion current (TIC) of all authentic phytohormones combining positive and negative scan. (B) XIC of positive scan of authentic plant hormones. (C) XIC of negative scan of authentic plant hormones. (D) (IAA) and (E) (BA), as well as (F) (CA) and (G) (SA) can be resolved under multiple MRM conditions. (H) (JA), (I) (ABA) and (J) (SA) are chromatograms of Arabidopsis samples. Phytohormones, identified by their peak numbers are: 1, zeatin; 2, GA_3; 3, ICA; 4, IAA; 5, BA; 6, ABA; 7, MeIAA; 8, SA; 9, MeBA; 10, CA; 11, IBA; 12, JA; 13, MeSA; 14, H_2JA; 15, MeCA; 16, MeJA; 17,GA_4; 18, MeH_2JA; and 19, OPDA.

variability in ion yield due to ion suppression. Hormone amounts were normalized to the mass of fresh plant tissue extracted.

To verify that peak area increased proportionally as a function of increasing hormone concentration (mol/L), solutions with various amounts of phytohormones (1 ng/mL to 2000 ng/mL) and fixed amounts of the corresponding internal standards (50 ng/mL) were analyzed. The dose–response curves were linear in the concentration ranges selected for various compounds (R^2 values of 0.916–0.999). Extraction efficiencies of endogenous phytohormones from plant samples were determined by extraction of each sample four times followed by LC–ESI–MS/MS analysis of each extract separately. The extraction efficiency was determined by comparing the peak area for each endogenous plant hormone in each of the four successive extractions. Typically no hormones were detected after two rounds of extraction. The extraction efficiencies of the hormones in this study ranged from 85% to 98% in one round of extraction. The recovery of each internal standard was calculated based on the ratio of peak area that was obtained from the extraction of internal standard spiked to plant samples and the peak area of the same amounts of internal standards measured directly. The recovery for each internal standard after one round of extraction was greater than 95%.

The quantification limit for each phytohormone was defined by the ratio of signal/background noise (S/N) > 10. Limit of quantification by this method, calculated based on the dose response curves prepared under partial or whole concentration range of 1–1000 pg/mL, varied from 0.01 to 10 pg/g fresh weight. The limits were 0.01–0.1 pg/g fresh weight for JA, MeJA, MeSA, SA, and ABA; 0.1–1 pg/g fresh weight for IAA, MeIAA, IBA, ICA, MeCA, GA_3, GA_4, OPDA, and zeatin; 1–10 pg/g fresh weight for BA, MeBA and CA. Such sensitivity

enables quantification of all hormones in plant tissues analyzed in this study.

2.4. Alteration of hormone levels in plants after wounding and pathogen infection

To test the feasibility of using this method to analyze plant processes, Arabidopsis plants were either mechanically wounded or challenged with the fungal pathogen *Botrytis cinerea*, followed by LC–ESI–MS/MS analysis. Fresh leaves (50–100 mg) were used for each extraction. Wounding, as inflicted by pressing with a hemostat several times across the midvein of fully expanded leaves, induced a large increase in JA (>500-fold 1 h after wounding; Fig. 3A). In comparison, the level of free OPDA, a precursor for the biosynthesis of JA, was much higher than JA before wounding, but the magnitude of the wound-induced increase in OPDA was smaller than that of JA. This result is consistent with previous reports in which jasmonates and OPDA were measured by GC–MS (Schmelz et al., 2003), although the GC–MS methodology employed did not distinguish JA from MeJA. Like JA, MeJA levels increased after wounding (Fig. 3B), but the MeJA level was only approximately 1/100 of that of JA 1 h after wounding. The temporal patterns of wound induction of JA and MeJA were similar; a higher level was detected at 1 h than at 6 h after wounding. The pattern of wound-induced increases in free OPDA is different from that of JA and MeJA, with the highest level at 6 h after wounding (Fig. 3A). The difference may suggest that free OPDA has metabolic and cellular functions besides being a precursor for JA synthesis. The severe wounding also induced multi-fold increases in ABA, SA, and ICA, but had no significant effect on the levels of MeSA, IBA, IAA, CA, BA, zeatin, or GAs (Fig. 3). It has been previously shown that wounding alone does not induce ABA accumulation, but instead that desiccation caused by wounding can lead to ABA buildup (Birkenmeier and Ryan, 1998).

B. cinerea is a necrotrophic fungal pathogen with a broad host range. Both JA- and SA-mediated signaling pathways have been implicated as being involved in plant response to *B. cinera* infection (Kachroo et al., 2003; Nandi et al., 2005). Inoculation with *B. cinerea* or a pathogen-free solution (control) induced a transient increase in the levels of JA 1 h after the treatment (Fig. 4). This increase was likely to result from minor wounding inflicted by three pricks with a 23 gauge needle used for inoculation. On the other hand, SA, MeSA, ABA, IAA, or BA showed no significant increase at 1 h after pricking (Fig. 4), but large increases in JA, OPDA, SA, and ICA were observed 24 h after *B. cinerea* inoculation (Fig. 4). The levels of MeJA, ABA, IAA, MeIAA, and MeSA also increased, but their fold increase was smaller than that of JA, SA, OPDA, and ICA. In contrast, infection by *B. cinerea* had no significant effect on BA or other hormones analyzed in the study (data not shown).

The levels of hormone esters MeJA, MeSA, MeIAA, MeBA and MeCA were substantially lower than their corresponding acids both before and after wounding or *B. cinerea* infection (Figs. 3 and 4). While there was a large increase in SA at 1 h after wounding, the level of MeSA was not changed. In contrast, wounding induced an increase in MeBA, but not BA. When plants were infected with *B. cinerea* for 12 h, large increases were observed in JA

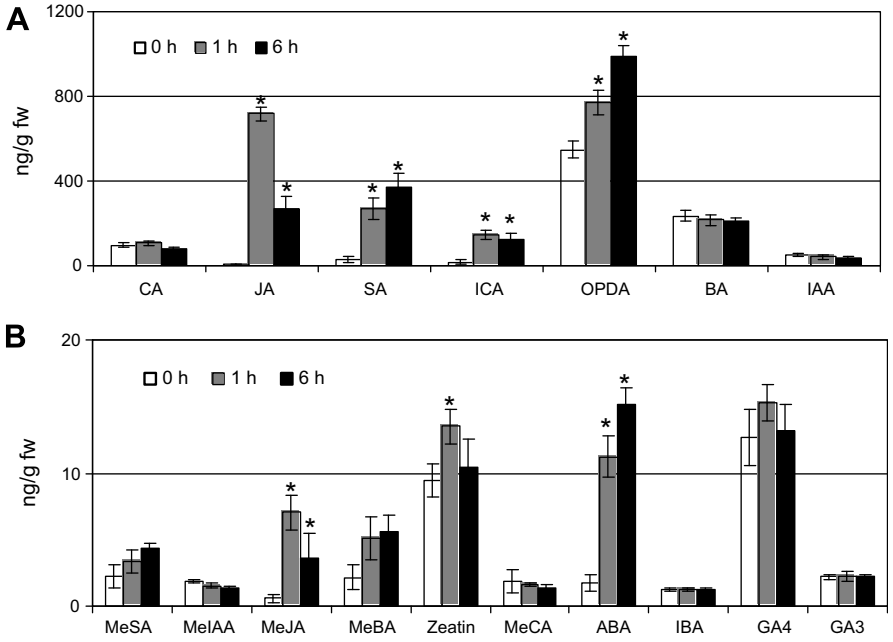

Fig. 3. Wound-induced changes in phytohormones and metabolites in Arabidopsis. Fully expanded leaves of Arabidopsis plants (4–5 weeks) were mechanically wounded by a hemostat several times across the midvein. Samples were collected at the time intervals indicated. Values are means ± SD ($n = 5$ independent sampling). The symbol "*" denotes a significant difference at $P < 0.05$ from unwounded leaves.

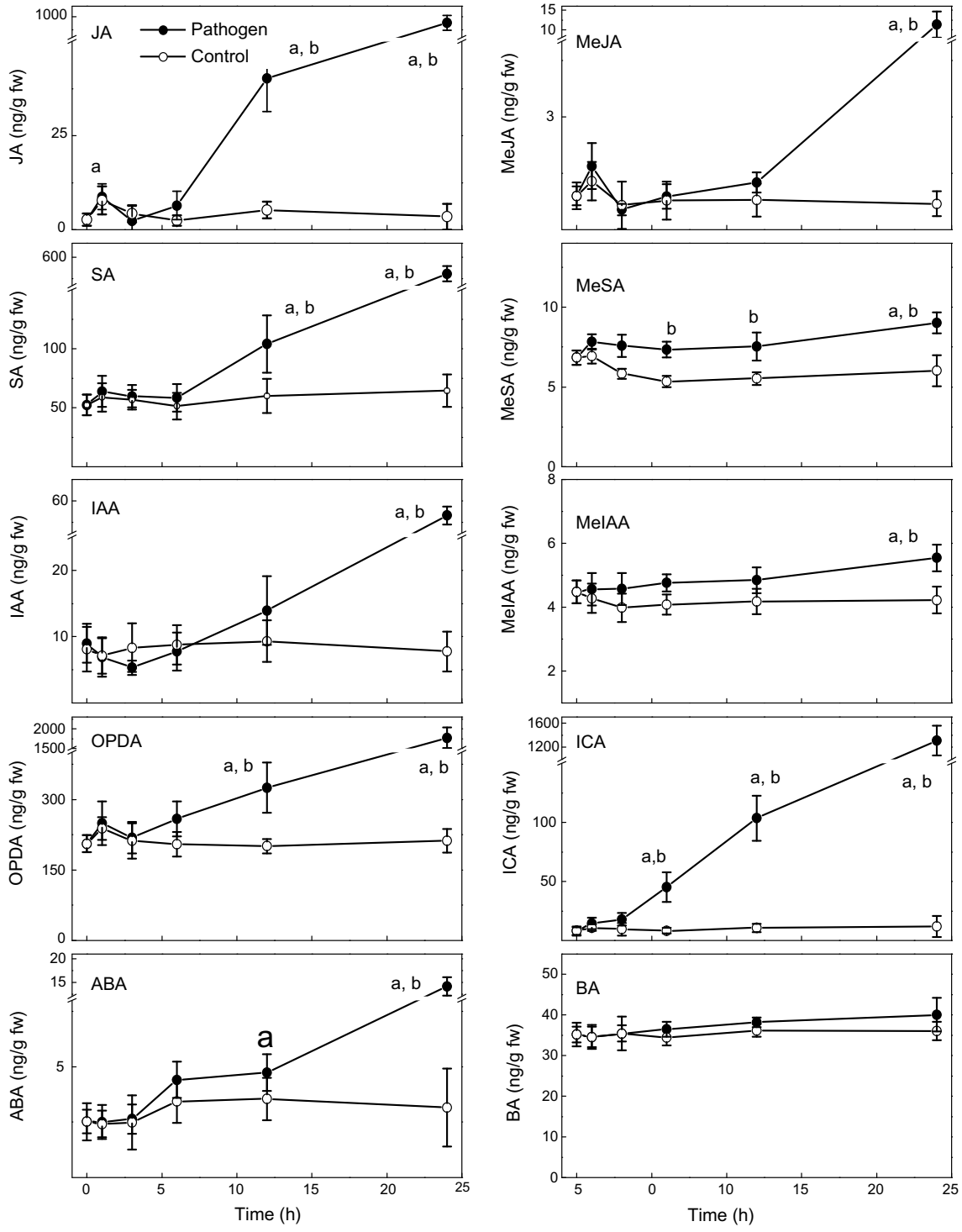

Fig. 4. Changes in phytohormones and metabolites in Arabidopsis after *Botrytis cinerea* infection. Arabidopsis plants (4–5 weeks) were inoculated with *Botrytis cinerea,* and leaves were harvested at different intervals after treatments. Values are means ± SD ($n = 5$ independent sampling). The letter "a" denotes a significant difference at $P < 0.05$ from samples at the initial time point of inoculation. The letter "b" denotes a significant difference at $P < 0.05$ from control (water-inoculated) samples at the same time points.

and OPDA, but not in MeJA (Fig. 4). At 24 h after inoculation, the levels of SA and IAA increased by 6-fold and 10-fold, respectively, but the increase in MeSA and MeIAA was modest, about 20%. Pathogen treatment altered the distributions of acid and methyl ester forms of SA, JA and IAA, but not BA and CA (Table 2). Ratios of acid to methyl ester forms of SA and JA were greatly increased (SA from 7.7 to 53.4, JA from 3.0 to 77.5) after 24 h of pathogen treatment. These differences in temporal patterns between hormone esters and corresponding acids suggest

Table 2
Ratios of acid to methyl ester forms of SA, JA, IAA, BA and CA in pathogen-treated Arabidopsis*

Hours	SA/MeSA	JA/MeJA	IAA/MeIAA	BA/MeBA	CA/MeCA
0	7.7 ± 1.5	3.0 ± 1.4	2.0 ± 0.4	16.9 ± 1.6	6.7 ± 1.1
1	8.2 ± 1.5	5.2 ± 1.6	1.6 ± 0.6	17.1 ± 0.4	5.7 ± 1.2
3	7.9 ± 1.7	4.3 ± 1.2	1.2 ± 0.5	18.9 ± 1.1	7.0 ± 1.1
6	8.0 ± 1.5	7.2 ± 1.3	1.6 ± 0.3	19.1 ± 0.4	4.6 ± 0.9
12	13.8 ± 1.9	32.1 ± 1.3	2.9 ± 0.9	19.8 ± 1.1	7.5 ± 0.8
24	53.7 ± 3.7	77.5 ± 5.3	9.6 ± 1.2	18.7 ± 0.6	6.5 ± 1.9

* Values are expressed as mean ratio ± SD ($n = 5$).

that the esters and acids may have distinguishable functions in defense responses.

Both wounding and pathogen infection induced significant increases in ICA, a metabolite in IAA catabolism (Figs. 3 and 4). ICA is regarded as a decarboxylative product of IAA and its formation occurs on a catabolic route to inactivate auxin function (Normanly, 1997). The initial concentration of ICA is lower than that of IAA, but after *B. cinerea* infection for 24 h, the level of ICA was approximately 20 times higher than that of IAA (Fig. 4). At 1 h after wounding, the level of ICA increased by 5-fold, but that of IAA was unchanged (Fig. 3). In addition, the level of ICA after wounding was almost 3-fold higher than that of IAA. The large increase in ICA and different patterns of ICA and IAA accumulation suggest that either there is a large stress-induced IAA biosynthesis and degradation, and/or a majority of ICA is made independently of IAA. Thus, it would be of interest to determine the function of the large ICA induction in plant stress responses.

The LC–ESI–MS/MS phytohormone analysis method reported here combines a simple extraction with a sensitive and accurate method for simultaneous quantification of multiple phytohormones from fresh plant tissues. The analyzed compounds belong to seven major classes of hormones and include a number of phytohormone metabolites; analyzed compounds include auxins (IAA, IBA, ICA, and MeIAA), cytokinins (zeatin), abscisic acid, gibberellins (GA_3 and GA_4), jasmonates (JA, MeJA, and OPDA), and salicylates (SA, MeSA, BA, MeBA, CA and MeCA). The LC–ESI–MS/MS method uses crude extracts without purification or derivatization and for quantification of most plant hormones in their natural states. The combination of retention time and monitoring of precursor ion-to-product ion transitions provides specific, sensitive, and accurate phytohormone determination. The method is versatile and would allow addition of other phytochemical analytes.

A strength of the method is the capacity to distinguish hormone esters from their acid forms in plants. Reports increasingly show that the hormone methyl esters play important roles in many plant processes. For example, MeJA and MeSA induce defensive responses and play roles in plant development processes, such as seed germination, flower and fruit development, leaf abscission, and senescence (Seo et al., 2001; Zhu and Park, 2005). Recently, MeIAA was found to regulate plant leaf development and auxin homeostasis (Qin et al., 2005). However, quantification of hormone methyl esters in plants has been difficult due to the low level and the limitations of analytical methods. We demonstrate here that this LC–ESI–MS/MS method provides adequate sensitivity for analysis of hormone methyl esters MeJA, MeSA, MeBA, MeCA, and MeIAA, and that the patterns of change in the methyl esters upon exposure to stress can be different from those of the respective acid forms. The differences suggest that methyl esters and their acids may have distinct functions in plant stress responses.

One person can easily prepare 80 samples for phytohormone LC–ESI–MS/MS analysis by the method described herein in 6 h. The most time-consuming steps are sample weighing, homogenization, and the LC–ESI–MS/MS analysis. Sample preparation can be hastened by using a tissue homogenizer (e.g., FastPrep FP 120, Qbiogene, Carlsbad, CA) to reduce homogenization time (Engelberth et al., 2003; Schmelz et al., 2003). Because the series of positive and negative MRM scans are looped together in this analysis, only one LC separation per sample need be performed, and 48 samples per day can be subjected to LC–ESI–MS/MS analysis if 30 min as reported here is used as per sample turn-around time. We have since been able to shorten the time to 20 min. Thus, sample preparation for this method is faster than some of the most efficient methods for phytohormone analysis previously described (Schmelz et al., 2003, 2004), and this method is more inclusive than those previously reported, in terms of classes of hormones quantitatively profiled (Chiwocha et al., 2003; Durgbanshi et al., 2005). The ability to distinguish hormone esters from their acid forms in plant extracts was not previously reported (Chiwocha et al., 2003; Durgbanshi et al., 2005; Schmelz et al., 2003, 2004).

The current analysis was aimed at providing a rapid, sensitive, and inclusive analysis for most hormone species. It should be noted that no one extraction procedure extracts structurally diverse compounds equally well. The reported acidic-organic phase extraction, in particular, may not recover basic compounds, such as cytokinins, very well. In this study, we used zeatin, one major class of cytokinins, as a target analyte and found that most of zeatin was partitioned into organic phase. In addition, a labeled zeatin was added during extraction as an internal standard that should have the same partition coefficient as endogenous zeatin. This helps to correct extraction efficiency and quantification. The one time, simple sample preparation also allows decreasing sample to sample variations, but proper sample handling is one key step to get reproducible results. In addition to using tissues of same age, size, and growth condition, as well as similar fresh weight, tissues need to be dipped into the liquid nitrogen immediately after collection and grounded into powder in the presence of liquid nitrogen. Solvents and internal standards were added to the powder while frozen.

3. Concluding remarks

We report a rapid, sensitive, and inclusive method for simultaneous quantification of phytohormones in native forms without purification or derivatization. This approach is applicable to analysis of dynamic changes of a broad range of phytohormones and related metabolites in plant response to abiotic and biotic stresses. The hormones analyzed represent structurally diverse compounds with different chemical properties. This approach can be expanded to simultaneously quantify multiple small biological molecules from complicated tissue matrices without purification and derivatization.

4. Experimental

4.1. Chemicals

Zeatin, gibberellins (GA_3 and GA_4), ICA, IAA, BA, ABA, MeIAA, SA, CA, MeBA, IBA, JA, MeSA, MeCA, MeJA, OPDA, dihydrojasmonic acid (H_2JA), and dihydrojasmonic acid methyl ester (MeH_2JA) were purchased from either Aldrich or Sigma Chemical Co. (St Louis, USA). Isotopically labeled internal standards including [$^2H_5$]BA, [$^2H_7$]CA, [$^2H_4$]MeSA, [$^2H_6$]SA, and [$^2H_5$]zeatin were purchased from Sigma Chemical Co. or OlChemim Ltd. (Olomouc, Czech Republic); [$^2H_5$]IAA and [$^2H_6$]ABA were purchased from ICON Isotopes (Summit, NJ, USA). The isotopically labeled ester internal standards including [$^2H_7$]MeCA, [$^2H_5$]MeBA, and [$^2H_5$]MeIAA were purchased from ICON Isotopes or obtained by methylation of corresponding isotopical standards. [$^2H_2$]GA_4 and [$^2H_2$]GA_3 were purchased from Dr. Lewis Mander of the Australian National University (Canberra, Australia).

4.2. Plant materials and sample preparation

Seeds of *Arabidopsis thaliana* wild-type (Columbia ecotype) were sown in Scotts Metromix 360 soil. The pots were kept at 4 °C for 2 days and then moved to a growth chamber at 23 °C (day) and 18 °C (night) with a 12-h day length, daytime fluorescent lighting at 120 μmol m^{-2} s^{-2}, and 58% relative humidity. Approximately 50–100 mg of fresh Arabidopsis leaves were sealed in 1.5 mL snap-cap vials. After being frozen in liq. N_2, the leaves were ground into powder, and 500 μL of 1-propanol/H_2O/concentrated HCl (2:1:0.002, vol/vol/vol) with internal standards (10–50 ng) were added, followed by agitation for 30 min at 4 °C. CH_2Cl_2 (1 mL) was added, followed by agitation for another 30 min and then centrifugation at 13,000*g* for 5 min. After centrifugation, two phases were formed and plant debris was in the middle of two layers. The lower layer (around 1 mL) was concentrated and re-solubilized in MeOH (0.3 mL) and then 25 μL was injected to column for analysis. The lower layer (25 μL) was also directly injected to column for analysis. Fungus strain *B. cinerea* was used for inoculation on 5-week-old soil-grown plants. Fungal spores were prepared and quantified as described (Broekaert et al., 1990). For inoculation, three needle-prick wounds were applied to each leaf, and the wounds were covered immediately with 5 μL drops of a suspension of 10^5 conidial spores per mL in potato dextrose broth medium (Difco). Inoculated plants were incubated at 18 °C under dim light at 100% relative humidity in propagator flats covered with a clear polystyrene lid for the time periods indicated. For mechanical wounding, fully expanded leaves were wounded by pressing with a hemostat several times across the midvein and then were kept under light as described previously (Wang et al., 2000).

4.3. Mass spectrometry

For selection of diagnostic precursor-to-product ion transitions, mixtures of 200 ng/mL of standard compounds dissolved in 50% MeOH with 0.1% HCO_2H were directly infused into a hybrid triple quadrupole/linear ion trap mass spectrometer (ABI 4000 Q-Trap, Applied Biosystems, Foster City, CA) outfitted with an electrospray (ESI) ion source using a 1 mL Hamilton syringe in a syringe pump at a flow rate of 1.2 mL/h. The analysis parameters were optimized for the production of characteristic precursor-to-product ion transitions in negative or positive ionization modes. SA, BA, IAA, IBA, ICA, ABA, CA, GA_3, GA_4, JA, OPDA, and their internal standards were scanned in the negative mode, while MeSA, MeIAA, MeCA, MeJA, MeBA, zeatin, and their internal standards were analyzed in the positive mode. The mixtures of standard compounds were separated by reversed-phase HPLC and analyzed by tandem mass spectrometry (RP-HPLC/ESI–MS/MS) in the MRM mode with 20 ms dwell time, 5 ms of pause time between mass ranges, and 700 ms of settle time for switching polarities.

The identities of phytohormones in the crude plant extracts were confirmed by analysis of product ion fragments obtained by the hybrid triple quadrupole/linear ion trap mass spectrometer, operating in the information dependent acquisition (IDA) mode, with a source voltage of 5.5 kV or −4.5 kV and source temperature of 300 °C. In the "Enhanced Product Ion" scan mode, precursor ions were fragmented with collision energy +25 kV or −25 kV and products in the *m*/*z* range of 50–500 were detected.

4.4. Reversed-phase high-performance liquid chromatography

Plant hormones were separated by an HPLC equipped with a reversed-phase column (C18 Gemini 5μ, 150 × 2.00 mm, Phenomenex, CA, USA) using a binary solvent system composed of water with 0.1% HCO_2H (A) and MeOH with 0.1% HCO_2H (B) as a mobile phase at a flow rate of 0.3 mL/min. Separations were performed using a gradient of increasing MeOH content. The initial gradient of methanol was kept at 30% for 2 min and increased linearly to 100% at 20 min.

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