The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites

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Summary

Through complex networks of signaling interactions, phytohormones regulate growth, development, reproduction and responses to biotic and abiotic stress. Comprehensive metabolomic approaches, seeking to quantify changes in vast numbers of plant metabolites, may ultimately clarify these complex signaling interactions and consequently explain pleiotropic effects on plant metabolism. Synergistic and antagonistic phytohormone signaling interactions, referred to as crosstalk, are often considered at the level of transduction without proper consideration of synthesis or accumulation of phytohormones because of the limitation and difficulty in quantifying numerous signals. Significant progress has recently been made in the expansion of metabolic profiling and analysis of multiple phytohormones [Birkemeyer et al. (J. Chromatogr. A, 2003, 993, 89); Chiwocha et al. (Plant J., 2003, 35, 405); Müller et al. (Planta, 2002, 216, 44); Schmelz et al. (Proc. Natl Acad. Sci. USA, 2003, 100, 10552)]. We recently presented a novel metabolic profiling approach to the analysis of acidic phytohormones and other metabolites based on a simplistic preparation scheme and analysis by chemical ionization-gas chromatography/mass spectrometry. We now provide a detailed description of this vapor phase extraction technique and use pathogen infection of Arabidopsis with Pseudomonas syringae DC3000 to illustrate metabolic changes in salicylic acid, cinnamic acid, jasmonic acid, indole-3-acetic acid, abscisic acid, unsaturated C18 fatty acids, 12-oxo-phytodienoic acid, and phytotoxin coronatine. Directions for further method expansion are provided and include issues of recovery, derivatization, range of accessible analytes, optimization, reproducibility and future directions.

Keywords: chemical ionization-gas chromatography/mass spectrometry, vapor phase extraction, metabolic profiling, phytohormone analysis, coronatine.

Introduction

A broad goal of plant genomics is to develop a comprehensive and systematic understanding of gene expression and function. In an effort to assign physiologic function to unknown genes, phenotypic analyses of morphologic, developmental and chemical traits are performed in mutant and transgenic plants. While informative, alterations in developmental and physical phenotypes may not precisely define the biochemical pathways affected. A much greater level of information is contained in a plant's complex chemical phenotype or metabolome. Similar to genomics,
yet technologically more daunting, the stated goal of metabolomic analyses is to indiscriminately identify and quantify all plant metabolites in an effort to understand gene function and interactions (Fiehn, 2002).

Advances in metabolomics and metabolic profiling efforts have been comprehensively reviewed (Fiehn, 2002; Sumner et al., 2003; Weckwerth, 2003). Showing significant promise for rapid adoption are electron ionization (EI) gas chromatography (GC) mass spectrometry (MS) profiling methods that enable the quantification of hundreds of compounds and spectral library-based identification (Fiehn et al., 2000a; Roessner et al., 2000, 2001a, b). Importantly, strategies for automated searches and identification of abundant plant metabolites using EI-GC/MS-based metabolite profiling have been reported (Wagner et al., 2003). These methods utilize simple lipophilic and polar extracts that are derivatized (transmethylated, methoximated, and trimethylsilylated) to increase stability and volatility enabling gas chromatographic separation. Highly abundant analytes readily profiled include many classes of primary metabolites such as amino acids, organic acids, sugars, fatty acids (FAs) and sterols. Of equal importance are liquid chromatographic (LC) approaches, in combination with universal detection such as MS, nuclear magnetic resonance and evaporative light scattering, which have the potential to be even more powerful given that separations do not require derivatization or volatilization and are well suited for large thermally labile analytes (Wolfender et al., 2003). Updated methods for LC metabolic profiling include saponins (Huhman and Sumner, 2003), phenolics (Chen et al., 2003), highly polar sugar derivatives (Tolstikov and Fiehn, 2002), and carotenoids (Fraser et al., 2000).

As a minor component of the metabolome, phytohormones are of particular significance given their role in the regulation of germination, growth, reproduction, and the protective responses of plants against stress (Davies, 1995). Phytohormones regulate metabolism through complex signaling networks often with interactive effects, referred to as crosstalk, with examples including plant responses to drought, wounding, and biotic stress (de Bruxelles and Roberts, 2001; Kunkel and Brooks, 2002; Zhu, 2002). For example, both positive and negative interactions on the expression of chemical and molecular responses have been described for JA-ethylene (E) (Kahl et al., 2000; Xu et al., 1994; Zhu-Salzman et al., 1998) SA-E (Berrocal-Lobo et al., 2002; O'Donnell et al., 1996, 2001, 2003), and SA-JA (Doares et al., 1995; Niki et al., 1998; Rao et al., 2000). It is now established that the coordinated interactions of multiple phytohormones control plant responses following numerous forms of stress (Reymond and Farmer, 1998). Likewise, complex sequences of phytohormone interactions have been described following pharmacologic treatments, pathogen infection, and seed thermomorancy (Chiwocha et al., 2003; Hansen and Grossmann, 2000; Veselov et al., 2003).

These results highlight the importance of metabolite analyses that monitor changes in multiple phytohormone pools. Current metabolomic approaches are able to quantify highly abundant primary and secondary metabolites but do not perform well at detecting trace levels of phytohormones. Separate profiling methods, with comparatively more elaborate sample preparation procedures, are now making phytohormone profiles accessible using trace analysis chemical ionization (CI) GC/MS techniques (Birkemeyer et al., 2003; Müller et al., 2002). Using LC/MS detection, a significant phytohormone profiling advance was recently achieved by Chiwocha et al. (2003). In this work, the authors simultaneously quantified free and conjugated forms of auxins, cytokinins, gibberellins, and abscisic acid (ABA) from thermomorant lettuce seeds and demonstrated a high level of metabolic activity. In contrast to LC approaches, volatile phytohormones and their derivatives, which include E, methyl salicylate (SA-ME), and methyl jasmonate (JA-ME), can be profiled using both static and dynamic headspace (HS) GC techniques without the need for tissue extraction or in some cases even liquid solvents (Harper, 2000; Kao and Yang, 1983; Kolb, 1999; Meyer et al., 2003; Raguso and Pellmyr, 1998; Seto, 1994). Static HS methods involve the direct analysis of an air sample drawn from a closed airspace containing the odor source. Advantages of simplicity are balanced by the lack of a pre-concentration step, which limits the application to analytes present at high concentrations (Kolb, 1999). Dynamic HS techniques enable the quantification of trace levels of analytes through the prolonged trapping, and thus concentration, of volatiles from a gaseous stream. Adsorbents are most commonly used for trapping and the sample is either thermally or chemically desorbed to release the analytes (Harper, 2000). Use of HS GC for VOC profiling continues to expand with over 6000 HS GC already in the literature (Snow and Slack, 2002).

To bridge the gap between highly specific target analyses and broader metabolomic approaches, the creation of simple methods for profiling a wide range of selectable analytes will be of great utility. Numerous separate methods have been created for the direct chemical quantification of individual phytohormones and regulators (Cornish and Zeevaart, 1985; Edlund et al., 1995; Mitchell, 1982; Moore et al., 1989; Mueller and Brodschelm, 1994; Turlings et al., 1991; Uknes et al., 1993). Modern GC/MS-based methods are now coming closer to inclusive phytohormone profiling and analysis (Birkemeyer et al., 2003; Engelberth et al., 2003; Müller et al., 2002; Schmelz et al., 2003; Weber et al., 1997). Distillation and dynamic HS methods represent mature techniques that enable the analysis of volatilized compounds via collection, concentration, and partial purification (Chaintreau, 2001; Heath and Manukian, 1994; Kolb, 1999; Lei et al., 2003; Raguso and Pellmyr, 1998). These widely utilized procedures predominate in research focused on flavors and fragrance, environmental toxicology, and
chemical ecology (Augusto et al., 2003; Cardé and Bell, 1995; Dewulf and Langenhove, 1999; Steinhart et al., 2000). JA-ME and SA-ME represent common volatiles that are widely utilized by the flavor and fragrance industry while the physiologic roles of JA and SA remain under intense investigation as plant defense regulators (Dong, 1998; Reymond and Farmer, 1998). Many methods for the direct chemical analysis of volatile esters and plant tissue levels of corresponding free acids share a common similarity: they are separated through gas-phase chromatography as methyl esters (ME). Methylation of carboxylic acid phytohormones is a widely adopted form of derivatization enabling GC analysis (Hedden, 1993). Curiously, despite the broad use of (i) HS methods for trapping and concentration of volatile SA-ME and JA-ME and (ii) the derivatization of free acid phytohormones to the corresponding MEs for analysis, a combination of these techniques has only recently been applied to the sample preparation and purification of acidic phytohormones from plant tissues.

We have recently described a sample preparation procedure, termed vapor phase extraction (VPE), for multiple phytohormones, VOCs, and other metabolites (Engelberth et al., 2003; Schmelz et al., 2003). This chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) profiling method uniquely combines established extraction, derivatization, dynamic HS trapping, and solvent desorption techniques to result in a simple yet robust profiling method for a wide range of analytes. We now present a detailed account of the method described in Schmelz et al. (2003) and include the following: sample extraction, derivatization, collection of analytes using VPE, and an example using Arabidopsis infected with the pathogen Pseudomonas syringae pv. tomato DC3000 (PstDC3000). Additional method development issues regarding recovery of low and high boiling point (bp) analytes, additional analytes and derivatives, reproducibility, and future directions are also discussed.

Results and discussion

Extraction

The knowledge of changes in endogenous metabolite levels over time is essential in addressing basic questions in phytohormone biology and plant metabolism. Direct chemical analysis requires the destructive harvesting of selected tissues with sample preparation decisions based on chemical properties of the analytes and the nature of the surrounding matrix (Smith, 2003). To minimize artifact generation, biological samples were frozen in liquid N2 and maintained at −70°C immediately prior to solvent extraction. Small leaves and tissue samples were processed directly while larger tissue samples were pulverized first in liquid N2 to generate a homogenous aliquot. In this work, we selected a FastPrep® bead mill (Qbiogene, Carlsbad, CA, USA), commonly used for RNA and DNA extraction, to efficiently and reproducibly lyse the plant material. Similar ball mills are increasingly used for homogenization and extraction of plant tissues in metabolomic and phytohormone analyses (Fiehn et al., 2000a; Müller et al., 2002). Weighed tissue (100 mg) was uniformly pulverized by the instruments rapid shaking action in the presence of ceramic beads, internal standards for quantification, and extraction solvent (Figure 1a). The homogenized material was then partitioned between MeCl2, briefly re-homogenized and centrifuged (Figure 1a). The top aqueous and lower MeCl2:1-propanol layers concentrate highly polar and lipophilic metabolites, respectively.

Efficient partitioning of acidic phytohormones from the aqueous to organic phase requires protonation of the carboxylic acids. For example, the recovery of SA into the organic layer is greatly reduced at a neutral pH; thus, a target pH of 3.0 was selected for the aqueous layer. Use of internal standards quantitatively corrects for potential pH-derived differences in extraction efficiency between samples. The acidified 1-propanol:H2O extraction mixture works well for many leaf tissues; however, additional HCl may be required for seeds, pathogen-infected tissues or samples otherwise rich in secondary metabolites. The use of additional acid can be empirically gauged by testing the aqueous layer with pH paper. Care should be taken to avoid excessively acidic conditions (pH < 2.0) as this can reduce indole-3-acetic acid (IAA) recovery. Similarly, additional water (200–300 µl) can be helpful in the extraction of dry tissues to aid in the exclusion of carbohydrates and highly polar metabolites from the organic phase. In contrast to other analytes, efficient recovery of JA appeared remarkably insensitive to numerous methodologic changes. For safety reasons, the use of combustible solvents in enclosed homogenization and centrifugation equipment was avoided. The above protocol represents the best compromises during 2 years of experimentation, yet may not be fully optimized. For example, development of a strongly buffered (pH = 3.0) extraction solution might alleviate pH-related extraction issues. Alternatively, a non-aqueous extraction of lyophilized material may eliminate the need for solvent partitioning and transfer.

Derivatization: production of methyl esters

To increase volatility and improve gas phase chromatography of carboxylic acid phytohormones, the organic phase was transferred to a 4-ml glass vial and derivatized to MEs directly using trimethylsilyldiazomethane (Figure 1b). The presence of 10–20% of a short chain alcohol is required for efficient ME formation when using trimethylsilyldiazomethane (Hashimoto et al., 1981). Based on the proposed reaction mechanism, the donated methyl group for ester formation originates from the trimethylsilyldiazomethane and not the added alcohol. We selected 1-propanol for the

initial extraction as it partitions into the dichloromethane with little transfer of water. In practice, 2 µl of a 2.0 M trimethylsilyldiazomethane solution represented twofold excess in reagent required for maximum ME production in 100 mg of extracted maize or Arabidopsis tissue and was thus standardized upon to for reproducibility. The activity of excess trimethylsilyldiazomethane was quenched with 2 µl of 2.0 M acetic acid in hexane (Figure 1b). After an incubation time of 30 min, for both the derivatization and quenching steps, VPE was performed. In cases where naturally occurring VOCs are not intentionally analyzed, the MeCl₂:1-propanol layer can be dried off under an N₂ stream and the samples resolubilized in a minimal amount of 1:9 MeOH:diethylether (100 µl) and derivatized as stated above. In this case, the use of dry solvents is required to ensure efficient derivatization. This method is routinely used when phytohormone profiling is of primary interest and results in a 50% reduction in time required by the VPE step by minimizing the volume of solvent that must pass through the system.

A limitation of this general approach is the lack of discrimination between MEs and free acids, as the two metabolic pools are combined during derivatization. A simple solution is to process an additional sample where ME internal standards are added, the derivatization step is bypassed, and only naturally occurring MEs are measured. Subtraction of the endogenous MEs from the total estimate would yield individual pool sizes. In most cases endogenous MEs of SA, JA, 12-oxo-phytodienoic acid (OPDA) are present at less than 10% of the free acid pools (Kramell et al., 2000; Seskar et al., 1998). Similarly, if naturally occurring VOCs pools are the primary analytes of interest, no derivatization is required.

**Vapor phase extraction**

In the analysis of both SA and JA, Engelberth et al. (2003) presented a simple 80°C VPE method that utilized a vacuum controlled flow of ambient air over the derivatized sample and through a collection filter containing the adsorbent, Super Q (Figure 2a). The volatile collection filters are constructed from inert materials including glass, fluorocarbons, and stainless steel mesh (see Experimental procedures). Super Q is a highly stable divinylbenzene polymer tolerant to H₂O vapor and sensitive only to temperatures above 300°C or the presence of strong oxidizing acids. To make use of these adsorbent properties and increase the range of analytes recovered, volatilization temperatures were increased from 80 to 200°C (Schmelz et al., 2003). This change necessitated the use of an N₂ stream, instead of ambient air, to reduce sample oxidation (Figure 2b).

The collection of VPE samples, inclusive of both acidic phytohormone MEs and VOCs, requires approximately 3–5 min (Figure 2c). Procedures for collecting a series of samples are as follows: (i) N₂ flow through the needle is initiated with excess pressure released to a bubbler with the available flow exceeding the vacuum when the system is fully connected. (ii) The 500 ml min⁻¹ flow generated by the
The Super Q filter is calibrated with a needle valve and periodically confirmed using a flowmeter. (iii) The Super Q filter is first inserted into the high temperature septa, followed by the N₂ stream needle, and finally the 6 Tygon/C₂₁₀ vacuum line (Saint-Gobain, Akron, OH, USA) is connected to the Super Q filter. Note: if the vacuum is accidentally connected first, the accumulated negative pressure in the vial will transiently exceed the capacity of the N₂ stream; thus, pulling water into the system from the positive pressure bubbler. Upon proper connection, solvent vapors pass through the Super Q filter and the trapping of volatile analytes is initiated. (iv) To expedite evaporation of the solvent, the connected vial (Figure 2b,c) is placed in a 70°C aluminum (Al) heating mantle until the solvent is evaporated, typically 2–3 min. Care should be taken to avoid temperatures that result in boiling as non-volatile components can contaminate the adsorbent. At this time, the dry vial was transferred to a second heating block at 200°C for 2 min. These elevated temperatures are required to aid in the recovery of less volatile compounds such as IAA-ME, ABA-ME and COR-ME. Once the VPE collection process is completed, the sample is disassembled by handling the gas lines and open top caps, with care taken to avoid touching the heated glass. For analysis, the Super Q filters are eluted into a sample vial with 150 μl of MeCl₂ and analyzed by CI-GC/MS.

With additional lines carrying the N₂ stream and vacuum, multiple samples can be processed at the same time using VPE. For purposes of clarity, Figure 2c illustrates only a single sample arrangement. We routinely collect acidic phytohormone-MEs as volatiles on four samples simultaneously. The VPE step requires approximately 2.5 and 5 min for small groups of samples derivatized in MeOH:diethyl-ether (100 μl) and MeCl₂:1-propanol, respectively. The rate-limiting steps of this procedure involve weighing the frozen plant material to be extracted and GC/MS run time. A single person can easily extract and process 48 VPE samples in 6 h period which equates to approximately 24 h of GC/MS analysis time. Thus, the primary limitation in the number of samples analyzed is based on the number of available CI-GC/MS instruments.

Mass of plant tissue required

Specialized methods for specific phytohormone analyses have been established enabling quantification from extremely low masses of plant tissue; for example, IAA measurements from ≤1 mg root and leaf tissue (Casimiro et al., 2001; Edlund et al., 1995). To estimate the mass of Arabidopsis tissue required using the VPE method we first established isobutane Cl-GC/MS detection limits of pure ME STDs. Highly linear and reproducible responses were achieved in the examined range of 10 pg to 1 ng per injection. Detection limits for benzoic acid (BA), SA, CA, JA, IAA, ABA, 18:3, OPDA, and COR were at or slightly below 10 pg (see Figure S1). In a previous standard addition experiment, highly linear and reproducible recoveries were established for these analytes; however, detection limits and minimal tissue requirements were not explored (Schmelz et al., 2003). Arabidopsis leaf tissue, consisting of a mixture of both healthy and PstDC3000 infected plants, was homogenized and extracted in amounts of 1, 10 and 100 mg. Analytes (ng g⁻¹ FW) in 100 mg tissue samples (n = 3) were estimated as follows: BA (474 ± 55), SA (168 ± 6), CA (16 ± 0.2), JA (936 ± 54), IAA (10 ± 0.5), ABA (18 ± 1), 18:1 (2738 ± 379), 18:2 (1768 ± 70), 18:3 (3305 ± 147), OPDA (1577 ± 59), and COR (118 ± 19). In the 10 mg tissue samples, IAA could not be reproducibly detected; however, all other analytes were readily quantifiable. In 1 mg tissue samples SA, CA, JA, ABA, 18:3 and OPDA were still detectable; however, many of these analytes were nearing a 3:1 signal-to-noise ratio and required manual integration. COR could not be detected in the 1 mg tissue samples. Unreasonably large peak areas for BA-ME in the 1 mg and blank tissue samples strongly suggested contamination of solvents with BA. Because of these artificially high backgrounds, control tissue levels BA-ME cannot be accurately reported. Most analytes displayed highly linear responses between 1 and 100 mg of tissue extracted (see Figure S2); however, to profile the widest range of acidic phytohormones, 100 mg FW of Arabidopsis tissue was selected. For specific phytohormone classes, such as the jasmonates, 10 mg of plant tissue would be sufficient for analysis.

Method comparisons: VPE versus SPE

Many GC/MS-based methods for phytohormone analyses utilize solid phase extraction (SPE) columns as initial purification steps prior to analyte derivatization. SPE matrices, often functioning as ion exchangers, are useful for the concentration of acidic phytohormones and aid in the removal of major components including lipids and chlorophyll (Edlund et al., 1995; Müller et al., 2002; Ribnicky et al., 1998). On the one hand, SPE procedures provide significant advantages in the selective and rapid exclusion of many chromatographically interfering compounds resulting in greatly increased sample purity and ultimately analytical sensitivity. On the other hand, a disadvantage of specialized SPE procedures is that this same selectivity excludes a broad range of analyte classes for inclusive metabolic profiling. In the described VPE protocol, metabolites are simply partitioned between aqueous and organic solvent layers. Using VPE, the purification and selectivity step takes place during volatilization with non-volatile components, having largely unfavorable gas-phase chromatographic properties, excluded. One the one hand, VPE can quantitatively recover VOCs and other derivatized analytes producing a rich GC-compatible sample for metabolic profiling. On the other hand, the VPE sample is complex which leads to an increased probability that some analytes may have high background signals from co-eluting compounds. This problem is largely, although not entirely, solved through the use of isobutane Cl and the production of predominant [M + H]⁺ ions with little if any associated fragmentation (see Figure S3). Using SPE columns, selectivity is determined by the column matrix and elution solvents chosen. In VPE, some selectivity is achievable through the choice of collection temperature and derivatization agents that influence the volatility and recovery of target analytes. Direct sample preparation comparisons using VPE and SPE columns have been performed for JA and SA (Engelberth et al., 2003). Overall, VPE produced very similar quantitative results with greater sensitivity. Direct comparisons with IAA, ABA, and free FAs have not been performed; however, the VPE results are consistent values reported in the literature.

Biologic samples: PstDC3000 infection of Arabidopsis

Arabidopsis infected with the bacterial pathogen PstDC3000 was used to illustrate the types of profiles generated using the VPE CI-GC/MS technique. For clarity, the reconstructed ion chromatogram was limited to 12 analytes (Figure 3) and thus represents a fraction of the information contained in the total ion chromatogram potentially useful for metabolic profiling. Defense signaling during Pseudomonas infection is complex with the interactions of E, SA, JA and polyketide phytoxins currently under investigation (Kunkel and Brooks, 2002; Thomma et al., 2001; Zhao et al., 2003). In Arabidopsis, basal resistance to PstDC3000 growth is dependent upon SA accumulation and signal perception (Dong, 1998; Nawrath and Metraux, 1999). Plants insensitive to the phytoxin COR display both greater increases in SA levels and increased resistance when infected with PstDC3000 (Kloek et al., 2001). Pathogen-produced phytoxins, such as COR, function not only as signal mimics (Weiler et al., 1994) but can also induce the production of E and JA (Kenyon and Turner, 1992; Laudert and Weiler, 1998). With structural similarity to OPDA, COR acts as a jasmonate analog and displays 100–1 000 times greater activity than JA in selected bioassays (Koda et al., 1996; Weiler et al., 1994).
1994). In Arabidopsis, exogenous COR application stimulates increased JA levels within 48 h (Laudert and Weiler, 1998). In tomato, PstDC3000 infection induces the expression of JA biosynthetic genes including LoxD, AOS1, AOC, and OPR3 (Zhao et al., 2003). Importantly, COR can function as a signal independent of JA levels in jasmonate biosynthetic mutants (Zhao et al., 2003).

In this experiment, pathogen infection with PstDC3000 resulted in average leaf COR levels of 1047 ng g⁻¹ FW at 96 h which is consistent with the established induction of JA biosynthetic transcripts and accompanied increases in both JA and OPDA (Figure 4a). COR-induced E production may further synergize jasmonate signal transduction as both phytohormones are required for wound-induced PIN gene expression in tomato (O’Donnell et al., 1996). COR is believed to compromise the production of pathogen defenses by triggering the massive production of jasmonate-induced anti-herbivore defenses thereby depleting resources available for defense and suppressing SA-based microbial resistance (Zhao et al., 2003). Despite the role of COR in disease progression and as a mediator of crosstalk interactions, direct chemical quantification of COR and multiple interacting phytohormones has only recently been demonstrated (Schmelz et al., 2003).

PstDC3000 induced increases in JA and OPDA at 48 h preceded the increase in unsaturated free FAs at 72 and 96 h (Figure 4b). In tomato, rapid wound-induced increases in free FAs parallel the induction of JA levels (Conconi et al., 2003).
Similar increases in JA levels are also associated with leaf senescence (He et al., 2002); thus the late time point increases in oleic (18:1), linoleic (18:2) and linolenic (18:3) acid may reflect cell death and senescence processes. FAs also have independent roles in mediating signaling interactions. In Arabidopsis, a mutation in the gene ssi2, a stearoyl-acyl carrier protein desaturase, results in decreased levels of oleic acid and impaired ability of JA to initiate the induced expression of the defensin gene PDF1.2 (Kachroo et al., 2003). The JA-induced expression of PDF1.2 in ssi2 mutants is restorable following exogenous application of oleic acid (Kachroo et al., 2001). Reduced chloroplastic levels of free oleic acid are also associated with abnormally high constitutive SA levels suggesting that the levels of free FAs may also mediate crosstalk between JA- and SA-dependent signaling pathways (Kachroo et al., 2003).

Infection of Arabidopsis with PstDC3000 resulted in increased levels of CA (Figure 4c) consistent with the previously established accumulation of phenylalanine ammonia-lyase (PAL) transcripts (Dong et al., 1991). Over the 4 day period, free SA displayed a four to 17-fold increase while free CA displayed four to eightfold changes (Figure 4c). In tobacco, the biosynthetic pathway for viral-induced SA accumulation is believed to be through PAL, CA, and benzoyl-glucose (Chong et al., 2001; Yalpani et al., 1993). In response to tobacco mosaic virus, tobacco exhibits very modest increases in free BA in comparison with the large induction of conjugated BA pools (Chong et al., 2001). Unlike BA, free CA pools exist at much lower levels and are not typically examined. In Arabidopsis, it is now clear that a CA-independent pathway, via isochorismate, is responsible for pathogen-induced SA accumulation (Wildermuth et al., 2001). Based on these results, quantitative relationships between CA and SA are not anticipated. In support of this paradigm, SA increases were detected 48 h before significant CA increases (Figure 4c). The inclusion of chorismic and isochorismic acid analysis has not yet been investigated; however, a method that enables SA precursors from both established pathways would aid in clarifying unresolved SA biosynthesis questions (Metraux, 2002).

In this study, PstDC3000 infection induced not only SA and JA levels, as expected, but also IAA and ABA (Figure 4d). Multiple pathogens, such as Pseudomonas savastanoi pv. savastanoi, colonize plants by producing phytohormone imbalances that result in the induced proliferation of plant tissues. These pathogens often harbor iaaM and iaaH genes that convert tryptophan to IAA via indole-3-acetamide (Hamill, 1993). An additional function of pathogen-induced IAA levels may be the inhibition of plant defense responses. Using inoculations with P. savastanoi strains exhibiting differential IAA and cytokinin production, Robinette and Matthysse (1990) demonstrated a link between pathogen-produced IAA and a decrease in the intensity of hypersensitive responses in Phaseolus vulgaris following subsequent inoculation with P. syringe pv. phaseolicola. Likewise, in the fungus Fusarium oxysporum, transgenic expression of the iaaM and iaaH genes resulted in increases in both IAA production and pathogen virulence measured by reduced seedling emergence (Cohen et al., 2002). Based on available sequence information, PstDC3000 does not appear to harbor the iaaM and iaaH genes; however, in a comprehensive screen of 57 Pst pathovars a majority of the cultures produced detectable levels of IAA despite lacking these genes (Glickmann et al., 1998). A separate IAA biosynthetic pathway through indole pyruvate has been described in the pathogen Erwinia herbicola (Brandl and Lindow, 1996). Independent of plant or pathogen origin, elevated IAA levels may mediate the outcome of plant–pathogen interactions.

Pathogen-induced levels of ABA have been described following infection with bacteria, viruses, and fungi (Kettner and Dorffling, 1995; Steadman and Pequignot, 1970; Wennham et al., 1986). In this experiment, PstDC3000 stimulated a 1.5–3.8-fold increase in ABA between 48 and 72 h (Figure 4d). In sitiens tomato mutants, impaired in the conversion of ABA-aldehyde to ABA, strong resistance to the necrotrophic fungal pathogen Botrytis cinerea was demonstrated (Audenaert et al., 2002). Importantly, susceptibility to B. cinerea was restored following exogenous application of ABA to sitiens plants and further increased in wild type plants. The molecular basis for this increased resistance in sitiens mutants corresponded with an increase in B. cinerea induced PAL activity and greater sensitivity of the SA-dependent defense pathway (Audenaert et al., 2002). In Arabidopsis, exogenous addition of ABA increased plant susceptibility to avirulent Pst1065; however, inoculation of the ABA mutants, ab1 and ab2, with PstDC3000 did not result in altered bacterial growth or disease phenotypes (Mohr and Cahill, 2003). Similar to bacterial-induced IAA, fungal pathogens have also been reported to synthesize ABA (Kettner and Dorffling, 1995). In this experiment, possible roles for pathogen-induced increases in ABA remain unclear.

Directions for method development

Recovery and breakthrough The advantages of vaporization and adsorbent trapping in sample purification also present limitations; namely, reduced recovery of both low and high boiling point (bp) analytes. Large volumes of gas can cause highly volatile analytes to travel through the adsorbent bed and escape in the outlet stream resulting in a process termed breakthrough. Using a range of hydrocarbons and Tenax TA as an adsorbent, Baya and Siskos (1996) demonstrated that breakthrough is dependent on the total volume gas passed over the adsorbent and independent over a wide range in flow rates. Breakthrough also depends on temperature, presence of molecules with different adsorbent affinity, and interactions with water (Dettmer and Engewald, 2002).

Divinyl benzene polymer adsorbents, such as Super Q and Porapak Q, very weakly retain low bp solvents such as MeCl₂. In this case, the breakthrough of MeCl₂, 1-propanol, diethyl ether and MeOH is essential for the method’s utility. The use of back-up adsorbent traps is advised when concern over the breakthrough of target analytes arises. Over the course of method development, little evidence was found for the breakthrough of selected analytes (Schmelz et al., 2003).

We empirically examined conditions leading to reduced recovery by trapping two straight chain alkane standard series with low (C₇-C₁₈) and high (C₁₈-C₃₈) bp with two different flow rates. The alkane series provided a simple means to address recovery over different bps; however, detection using GC-FID was utilized as these compounds are not readily ionized using isobutane-Cl. Under both 50 and 500 ml min⁻¹ flow rates for trapping, significant breakthrough was detected for C₇-heptane (99°C bp) (Figure 5a). Recoveries of heptane at 50 and 500 ml min⁻¹ were 59.4 ± 2.9 and 1.9 ± 0.4, respectively. In the case of C₈-octane (bp 126°C), significant breakthrough was only detected at 500 ml min⁻¹ and not 50 ml min⁻¹ (Figure 5a).

Alkanes C₉ through C₁₈, representing bp ranging from 152 to 316°C, all displayed recoveries of nearly 100% under both flow rates. As expected, an opposite trend was demonstrated for high bp analytes. Recovery of the C₁₈-C₃₈ alkane series was significantly lower at 50 than 500 ml min⁻¹ (Figure 5b). At 500 ml min⁻¹, recovery for the C₂₆ (412°C bp) and C₃₈ (497°C bp) alkanes was 88 and 72%, respectively. Comparatively, the recovery of the same analytes at 50 ml min⁻¹ was 10–20% lower. Using the standard conditions (500 ml min⁻¹, 200°C, 2 min) these alkane-based results demonstrate that analyte bp should fall between 150 and 500°C for volatilization recoveries of 70% or greater.

Two additional experiments were performed on biologically relevant analytes. A series of C₆-C₄₄ FA-MEs, with bps ranging from 150 to 419°C, were spiked into plant tissue samples and recollected from the underivatized organic phase. Using the standard collection protocol, 500 ml min⁻¹ at 200°C for 2 min, C₆-caproic acid-ME (149.8°C bp) demonstrated a significant reduction in recovery (47.0 ± 3.3) because of breakthrough as predicted from the alkane results. Encouragingly, all other C₇-C₄₄ FA-MEs including caprylic (96.0 ± 5.4), capric (89.5 ± 8.0), undecanoic (89.0 ± 8.6), lauric (90.2 ± 8.6), tridecanoic (91.7 ± 9.9), myristoleic (93.2 ± 10.7), myristic (92.1 ± 10.2), pentadecanoic (93.0 ± 10.3), cis-10-pentadecenoic (95.3 ± 10.8), palmitic (92.5 ± 9.7), palmitoleic (94.5 ± 10.5), heptadecanoic (91.3 ± 10.5), cis-10-heptadecenoic (94.5 ± 11.2), linoleic (94.0 ± 11.7), oleic (90.0 ± 9.0), cis-11-eicosenoic (86.5 ± 12.0), heneicosanoic (86.5 ± 12.9), behenic (87.0 ± 12.6), tricosanoic (87.7 ± 12.8), lignoceric (87.4 ± 13.5), demonstrated recoveries of 87% or greater suggesting the method is well suited for profiling free FAs following derivatization to MEs. Total recoveries of spiked analytes from a plant matrix were previously determined for SA (50%), JA (80%), IAA (86%), ABA (68%), and COR (57%) (Schmelz et al., 2003). This includes losses that occurred during the extraction, solvent partition, derivatization, volatilization, and elution steps. The nearly 90% recovery of all spiked FA-MEs and 80% total recovery of the JA free acid suggests that the trimethylsilyldiazomethane derivatization performs at a high level of efficiency despite the relatively complex matrix of extracted plant material. Naturally occurring VOCs such as indole, β-caryophyllene, and α-humulene, that do not require derivatization were previously demonstrated to have 93–100% recoveries (Schmelz et al., 2003).

Using a flow rate of 500 ml min⁻¹, the effect of temperature and length of collection time was briefly explored with a mixture of β-pinene, (Z)-3-hexenyl acetate and SA-ME, JA-ME, and ABA-ME. At 30°C for 2 min, recovery levels closely matched the analytes’ bp (Figure 6a). Increasing the time of collection to 6 min at 30°C, aided in the recovery of all analytes except ABA-ME (bp 408°C) which was undetectable (Figure 6b). An increase in collection temperature to 220°C enabled maximal recovery of all six analytes within 2 min (Figure 6a); however, extending the collection period to 6 min resulted in reduced recovery of the lowest bp analyte, β-pinene (bp 166°C) (Figure 6b). The 20% loss of β-pinene under these extreme conditions is suggestive of breakthrough; however, thermal decomposition cannot be entirely ruled out (Cao and Hewitt, 1993). Extreme conditions can also result in degradation artifacts derived from adsorbents, similar to Super Q, and are known to include benzaldehyde, phenol, and n-aldehydes (Helmig and Greenberg, 1994). Thus, special attention is required when considering the analysis of these compounds. The single use and disposal of porous polymer adsorbents has been...
because of signal reduction via fragmentation or spectra library matching. In addition, in scanning EI peaks per chromatogram have been identified using EI this sample richness, very few of the approximately 300 GC equipped with a flame ionization detector (FID). Despite corn (Schmelz et al., 2003), are quantifiable using simply a CI-SIM-based quantification, by design, has low resolution and is susceptible to co-eluting compounds with similar or identical molecular weights. Actual confirmation requires either clear EI spectra or MS/MS daughter ion analysis. While virtually eliminating fragmentation and maximizing the \([M + H]^+\) ion signal, CI-GC/MS in full scan mode does not result in sufficient sensitivity for many of the low abundance phytohormones. Thus the ability to monitor both high abundance analytes and trace phytohormone levels is partially limited by the necessity to switch between SIM and full scan mode.

Additional analytes and derivatives. The FA-ME recovery experiment indicated that nearly complete free FA profiles should be accessible. FAs serve a multitude of roles, ranging from components of hydrophobic membrane barriers to precursors of active oxylipin signals (Blee, 1998, 2002). In the current design, MEs of oleic, linoleic and linolenic acid co-chromatograph yet they are readily resolved as \([M + H]^+\) parent ions (Figure 8a). In addition to linolenic acid, active cyclopentane oxylipins can also be derived from 7,10,13-hexadecatrienoic acid (16:3) resulting in the production of a 16-carbon analog of OPDA, termed dinor-OPDA (Weber et al., 1997). Both OPDA and dinor OPDA function as defense signals in the absence of JA (Stintzi et al., 2001) and are accessible through the VPE technique (Figures 3 and 7b). Additional JA-related metabolites, including cucurbitic acid (CU) and tuberonic acid (TA), have been isolated in screens monitoring growth inhibition (Fukui et al., 1977) and induction of potato tuberization (Yoshihara et al., 1989). Unlike JA, the physiologic roles of CU and TA in planta have yet to be conclusively demonstrated and remain largely overlooked in oxylipin analyses. Acylation of the hydroxyl groups results in only small changes in bp and ability to co-chromatograph yet they are readily resolved as \([M + H]^+\) parent ions (Figure 7b) (Wells, 1999). Following derivatization to MEs and acylation of alcohols using acetyl chloride, hydroxy FAs including ricinoleic (18:1-OH), 16-hydroxyhexadecenoic (16:0-OH) and 17-hydroxylinoleic (18:2-OH) are also accessible as ME-acetates (Figure 7c). Less stable lipoxynase (LOX) products such as, 13-hydroxy-9,11,15-octadecatrienoic acid (13-HOTE) are readily derivatized to MEs using trimethylsilyldiazomethane but undergo degradation upon acylation. VPE analysis of labile hydroxy FAs, such as

suggested (Harper, 2000); however, in this design the constructed Super Q filters are routinely reused and simply rinsed with MeCl2 prior to each use. The traps are easily constructed Super Q filters are routinely reused and simply

Accessing additional information

Figure 4 illustrates changes in 10 analytes during PstDC3000 infection of Arabidopsis; however, a much greater number of analytes should be accessible. Preliminary EI spectra investigations of tomato, corn, and tobacco samples have confirmed the presence of significant amounts of plant VOCs such as terpenes, nicotine, indole and numerous carboxylic acids. CI-GC/MS is essential to resolve trace analytes; however, these dominant metabolites (µg g⁻¹ FW), such as insect-induced sesquiterpenes in corn (Schmelz et al., 2003), are quantifiable using simply a GC equipped with a flame ionization detector (FID). Despite this sample richness, very few of the approximately 300 peaks per chromatogram have been identified using EI spectra library matching. In addition, in scanning EI mode, some phytohormones may be entirely undetectable because of signal reduction via fragmentation or co-chromatography with other compounds. Thus far, our most successful approach has been to consider the characteristics of target analytes and ask a series of questions regarding GC column compatibility (functional groups, molecular weight, bp), availability of standards, and ionization efficiency using isobutane CI. Armed with the above information the analyte presence is readily investigated in VPE samples using very sensitive selected ion mode (SIM) of the profile. In this approach, caution is also required as the CI-SIM-based quantification, by design, has low resolution and is susceptible to co-eluting compounds with similar or identical molecular weights. Actual confirmation requires either clear EI spectra or MS/MS daughter ion analysis. Currently, virtually eliminating fragmentation and maximizing the \([M + H]^+\) ion signal, CI-GC/MS in full scan mode does not result in sufficient sensitivity for many of the low abundance phytohormones. Thus the ability to monitor both high abundance analytes and trace phytohormone levels is partially limited by the necessity to switch between SIM and full scan mode.

13-HOTrE-ME, was enabled by switching the GC injector from split/splitless to a cool on-column arrangement (data not shown). While the biologic function of 13-HOTrE remains unclear, it is curious that SA treatment of barley leaves results in large accumulation of this LOX product (Weichert et al., 1999).

Ideally a single derivatization step could be used to protect a wide array of functional groups, increasing the

Figure 7. Vapor phase extraction (VPE) and chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) selected ion chromatograms of FA-MEs, oxylipin-MEs, and hydroxyoxylipin-ME-acetates (Ac).

(a) [M + H]+ ion separation of co-chromatographing steric (18:0; 15.80 min), oleic (18:1; 15.56 min), linoleic (18:2; 15.51 min), and linolenic (18:3; 15.53 min) acids.

(b) Jasmonates including cucurbic acid (CU)-ME-Ac (13.42 min), tuberonic acid (TA)-ME-Ac (14.98 min) and dinor-OPDA-ME (15.43 t 15.73 c min).

(c) Hydroxy fatty acids including 16-hydroxyhexadecanoic acid (16:0-OH)-ME-Ac (17.33 min), 17-hydroxylinoleic acid (18:2-OH)-ME-Ac (18.03 min). For each analyte, 1 μg was derivatized to the corresponding ME using trimethylsilyldiazomethane, additional acylation was performed on hydroxy analytes resulting in the ME-Ac. [M + H]+ parent ions and useful diagnostic ions (bold) are listed in brackets [ ].

Figure 8. Vapor phase extraction (VPE) and chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) of TMS [M + H]+ parent ions of phytohormone derivatives including ACC (2 TMS-246), SA (1 TMS-211), JA (1 TMS-283), IAA (2 TMS-320), 2iP (1 TMS-276), ABA (1 TMS-319), linolenic acid (1 TMS-351), OPDA (1 TMS-tc-365), Z (1 TMS-292), COR (1 TMS-392), and GA7 (2 TMS-475). The predicted 475 [M + H]+ ion for 2-TMS GA7 displayed relatively low signal intensity relative to the abundant 341 ion fragment. The x-axis denotes GC retention times and selected [M + H]+ m/z ions used in drawing the ion chromatogram. VPE conditions were 500 ml min−1 at 200°C for 2 min.
range of metabolites profiled and also minimizing sample handling and artifact formation. In theory, based on bp limitations (Figure 5), a majority of the non-conjugated phytohormones should be accessible using modifications of the VPE technique. For the purposes of phytohormone profiling and simultaneous analyses, Birkemeyer et al. (2003) have recently examined a wide array of derivatization techniques and describe yield, response ratios and utility. In this study, N-methyl-N-(tert. butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was determined to be most useful and produced tert.-butyldimethylsilyl (TBS) derivatives of 1-aminocyclopropane-1-carboxylic acid (ACC), IAA, JA, SA, ABA, and zeatin (Z). The primary advantage of silylation over other derivatization techniques is the protection of not only carboxylic acids but also alcohols and amines (Little, 1999). To explore the feasibility of using similar silyl derivatives in the VPE technique, we have recently begun working with BSTFA and monitoring the trimethylsilyl (TMS) derivatives. Using a combination of pure standards, TMS derivatives of ACC, SA, JA, IAA, N\(^2\)-(2-isopentenyl)adenine (2iP), ABA, 18:3, OPDA, Z, COR and giberellin A\(_1\) (GA\(_1\) ) were collected as volatiles on Super Q and detected by CI-GC/MS (Figure 8). This method has the potential advantage over ME formation using trimethylsilyldiazomethane, in that the E precursor ACC, cytokinins (2iP and Z), and some giberellins (GA\(_1\) ) may also be detected; however, increased richness of recovered analytes may come at a cost. The potential for co-chromatographing peaks and ions interfering with target analyte quantification is expected to increase in biologic samples. Another TMS issue is the stability of the derivatives in the presence of trace amounts of water and production of artifacts (Little, 1999). Numerous modifications will likely be needed, including drying of the sample prior to derivatization that, in turn, results in the loss of naturally occurring VOCs. Using the current sample preparation protocol, ACC will remain in the aqueous layer and similarly cytokinins have low solubility in MeCl\(_2\). An alternative derivatization worth considering is the highly stable permethylation of alcohols and amines, a technique previously used for giberrellins and cytokinins (Rivier and Crozier, 1987). The potential for routine tissue sample analysis of highly polar, non-volatile and thermally labile compounds, including the brassinosteroids, cytokinins, giberrellins and numerous conjugates, using VPE-CI-GC/MS profiling remains uncertain and likely problematic. Unquestionably, VPE will not be adaptable to all classes of plant regulators; however, recent trials with large molecules, such as sucrose octaacetate (bp 678\(^\circ\)C) indicate that numerous problems may arise at the GC inlet and not the VPE sample preparation step. Clearly, numerous modifications are worth exploring to broaden the range of phytohormones and metabolite classes profiled using VPE.

Reproducibility and utilization efficiency

Long-term chromatographic reproducibility with stable analyte retention times was achieved using two identical HP-1MS columns. The first column was used for chromatography while the second was used as a source of replaceable 1 m sections of ‘pre-column’ between the injector and analytical column. Glass press-fit connectors (Agilent, Palo Alto, CA, USA) were used to connect the pre- and analytical columns. In practice the ‘pre-column’ was replaced as needed based upon increased chromatographic tailing of compounds containing alcohols (SA-ME) and amines (IAA-ME), normally every 250–300 samples. The use of a ‘pre-column’ has resulted in minimal change of analyte retention times, over an extended period (for up to a year), which in turn has afforded significant time savings by minimizing the need for repeated adjusting of peak integration and quantification parameters. When using isobutane as a reagent gas, the MS CI source periodically requires cleaning and removal deposits. To minimize instrument downtime we also utilize two identical CI ion sources, so that the spare can be cleaned as time allows.

Future directions

A challenge and increasing interest in phytohormone analysis is the specific localization of phytohormone gradients and dynamic changes. Using an ion-exchange-based sample purification, multiple derivatizations using diazomethane and BSTFA and a double-focusing magnetic sector tandem MS, Edlund et al. (1995) were able to measure 500 fg of IAA from 1 mg samples of tobacco leaves. This exquisitely high sensitivity and low tissue requirement has also been utilized to map IAA concentration gradients in lateral meristem of Pinus sylvestris and the root tips of Arabidopsis seedlings (Casimiro et al., 2001; Uggla et al., 1996). Similarly, dramatic IAA kinetics over the course of development in individual carrot embryos have been described (Ribnicky et al., 1998, 2002). Examples of such specialized techniques represent state of the art micro-scale phytohormone analyses. One approach to analyzing small amounts of tissue is to minimize the amount of unutilized sample. In the current VPE design, 10–100 mg of plant tissue is extracted and in the final sample 1 of 150 \(\mu\)l is injected onto the CI-GC/MS. Using a modified VPE approach, 100-fold reduction in the amount of plant tissue required should be achievable with a non-diluting sample recovery process, such as thermal desorption (Harper, 2000). A greater distance between the volatilized sample and volatile trap will likely be required to lower the gas stream and adsorbent temperature enabling absorption of volatiles. Detailed breakthrough volumes and temperatures for a wide range of solvents and analytes on the thermally desorbed Tenax TA is available from Scientific...
Instrument Services (Ringoes, NJ, USA) and may serve as an excellent starting point. While requiring investment in specialized desorption equipment this modified approach may enable tissue and cell type-specific questions to be addressed. For example, localized phytohormone or phytotoxin levels at the site of pathogen-induced necrosis or changes within individual germinating seed tissues.

Concluding remarks

We present a simple metabolic profiling method for numerous phytohormones and metabolites. Importantly, the data presented represent a fraction of the potential information contained in the ion chromatograms, with potentially broad method applicability to low molecular weight analytes. Acquisition of appropriate internal standards and choice of derivatization agents are the primary limiting factors in the expansion of this technique. Genomic and microarray-based approaches are greatly increasing our knowledge of phytohormone-mediated regulation at the mRNA level following pharmacologic applications (Schenk et al., 2000). This work along with other recent advances in the profiling of phytohormones (Birkemeyer et al., 2003; Chiwocha et al., 2003; Müller et al., 2002) will enable a thorough investigation of numerous phytohormone interactions that occur as plants balance growth, homeostasis, and reproduction with protective responses against stress. Broad metabolic profiling approaches that analyze quantitative changes in primary metabolites, phytohormones, phytotoxins and secondary metabolites will facilitate the regulatory interpretation of dynamic transcriptional changes in a holistic manner.

Experimental procedures

Chemicals

Reagents and solvents including methanol (MeOH), ethanol (EtOH), diethyl ether, dichloromethane (MeCl2), acetic acid, HCl, 1-propanol, trimethylsilidiazomethane, acetyl chloride, and N, O-bistrimethylsilyl) trifluoroacetamide (BSTFA) were from Sigma-Aldrich (St Louis, MO, USA). Carboxylic acids and corresponding ME including 1-aminocyclopropane-1-carboxylic acid (ACC), benzoic acid (BA), methyl benzoate (BA-ME), salicylic acid (SA-ME), trans-cinnamic acid (CA), methyl trans-cinnamate (CA-ME), jasmonic acid (JA), methyl jasmonate (JA-ME), indole-3-acetic acid (IAA), indole-3-acetic acid methyl ester (IAA-ME), abscisic acid (ABA), (2,6,6-trimethyl-2-cyclohexene-1-carboxylic acid (TCHCA), 2-hexenoyl-3-carboxylic acid, and C7-C18 saturated chain alkanes were purchased from Sigma-Aldrich. Methyl dihydrojasmonate (Bedoukian Research Inc., Danbury, CT, USA) was subjected to alkaline hydrolysis to yield dihydrojasmonic acid (dHJA). Free FA and ME standards for caproic, caprylic, capric, undecanoic, lauric, tridecanoic, myristic, myristoleic, pentadecanoic, cis-10-pentadecenoic, palmitic, palmitoleic, heptadecanoic, cis-10-heptadecenoic, linoleic, linolenic, oleic, stearic, cis-11-eicosenoic, heneicosanoic, behenic, tricosanoic, lignoceric, and 16-hydroxyhexadecanoic acid were purchased from Sigma (St Louis, MO, USA). Ricinoleic acid was acquired from Nu-Chek Prep (Elysian, MN, USA). Cucurbic acid and 12-oxo-phytodienoic acid (OPDA) were purchased from Cayman Chemical (Ann Arbor, MI, USA); 17-hydroxylinolenic acid was synthesized as described in Alborn et al. (2000). Coronatine (COR), tuberonic acid, and dinor-OPDA were received as kind gifts from Drs Toshima (Ibaraki University, Japan), Kitahara (University of Tokyo, Japan), and Weber (University of Lausanne, Switzerland), respectively. Isotopically labeled internal standards including 1H2-IAA, 2H2-CA, 3H2-SA, 4H2-BA were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada), 13C6-BA and 2H2-ABA were from ICON Isotopes (Summit, NJ, USA), and 13C15-linolenic acid was from Martek Biosciences (Columbia, MD, USA). All estimates of analyte boiling points (bp) at 760 Torr were derived from the Advanced Chemistry Development Software Solaris V4.67 (ACD Inc., Toronto, Canada).

Sample preparation

Plant tissues were frozen in liquid N2 to halt enzymatic activities and stored at −80°C prior to use. While frozen, samples were weighed to approximately 100 mg and transferred to 2 ml screw cap FastPrep® tubes containing 1 g Zirmil® beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA). DhJA and isotopically labeled internal standards (100 ng each in 5 µl EtOH) were added to the 2 ml tubes prior to sample addition. The samples were extracted with 300 µl of 1-propanol:H2O:HCl (2:1:0.005) and shaken for 30 sec in a FastPrep® FP 120 tissue homogenizer (Qbiogene). One milliliter of MeCl2 was added to each sample, re-shaken for 5 sec in the homogenizer, and centrifuged at 11,300 × g for 30 sec. The bottom MeCl2:1-propanol layer was pipetted, with care taken to avoid mixing layers, and transferred into a 4 ml glass vial and sealed with a Teflon lined screw cap (SUN SRI; Wilmington, NC, USA; 200-492, 200-588).

Derivatization

Carboxylic acids in the MeCl2:1-propanol layer were converted to MEs by the addition of 2 µl of a 2.0 mM solution of trimethylsilyldiazomethane (4 µmol) in hexane. The vials were capped, vortexed, and allowed to sit at RT for 30 min. Excess trimethylsilyldiazomethane was quenched by adding an equivalent molar amount of acetic acid (approximately 4 µmol) to each sample and allowing 30 min for the reaction. Acylation was performed on hydroxy-FA and hydroxy-oxylipin standards to improve volatility, gas chromatographic behavior and applicability to VPE. Acylation of previously formed MEs of cucurbit, tuberonic, ricinolenic, 16-hydroxyhexadecanoic and 17-hydroxylinolenic acid was achieved by dissolving 1 µg of each compound in 50 µl acetyl chloride:MeCl2 (1:4) and incubating at 90°C for 10 min. Trimethylsilylation was performed on a mixture containing 1 µg each of ACC, SA, JA, IAA, iP, ABA, linolenic acid, OPDA, Z, COR and GA3 by incubating the sample in 50 µl of BSTFA at 100°C for 1 h. For both acylation and trimethylsilylation, remaining solvent and derivatization agents were removed under an N2 stream and the samples were re-solubilized in 100 µl MeCl2 prior to VPE.

Collection of analytes using Super Q filters and VPE

Target analytes, such as acidic phytohormones and naturally occurring VOCs are separated from the largely non-volatile matrix by VPE. To achieve this, Teflon-lined caps from the 4 ml vials were replaced with open top caps (SUN SRI; Wilmington, NC, USA; 200-492, 200-588)
fitted with high-temperature low-bleed Thermogreen® septa (11 mm; Supelco, Bellefonte, PA, USA). Volatilized analytes were trapped on approximately 30 mg of Super Q (80/100 mesh) adsorbent (Alltech Associates, Inc., Deerfield, IL, USA). The Super Q filters were constructed from the following: perfluoroalkoxy fluorocarbon (PFA) tubing (5/32 ID × 1/4 OD; Cole Parmer Instrument Co., Vernon Hills, IL, USA), inner borosilicate glass collection tubing (Kimax® KG-33 standard wall tubing, 4 mm OD, wall 0.8 mm; Kimble-Kontes, Vineland, NJ), stainless steel mesh no. 325 filter discs to secure the adsorbent (Small Parts, Inc., Miami Lakes, FL, USA), and a 3/16" Mc Gill utility hole punch (Alpine Imports, Rockford, IL, USA) to cut the stainless steel mesh to the proper size. The inner glass and outer PFA tubing fit tightly together thus fire polishing of the glass rod ends and use of small press may be helpful in fitting the pieces together. Manufactured Super Q filters and related volatile collection materials are also commercially available (Analytical Research Systems, Inc., Gainesville, FL, USA).

For VPE, the Super Q filter was first inserted through the high temperature septa followed by a Perfectum® 18 gauge stainless steel needle (Thomas Scientific, Swedesboro, NJ, USA) carrying a low pressure N₂ stream buffered with a pressure release bubbler to allow changes in flow rate. The transfer line, between the N₂ cylinder and the VPE sample, was constructed from relatively inert materials, such as copper and fluorocarbon tubing. Highly flexible corrugated FEP tubing (7/32 × 1/4", 06407-60; Cole Parmer Instrument Co.) was preferred over Tygon tubing because of the absence of contaminating phthalates. An N₂ flow rate of 500 ml min⁻¹ through the trap was created with a corrosion-proof diaphragm vacuum pump (KNF Neuberger, Inc.; Trenton, NJ, USA) regulated and calibrated with a low-volume needle valve (JAX Valve & Fitting Co., Jacksonville, FL, USA; SS-4MG) and flow meter (Aalborg Instruments & Controls, Orangeburg, NY, USA; PMR-011806). The order of events are important and include first the insertion of the Super Q filter, then the N₂ stream needle, and lastly connection of the Tygon® vacuum line (ID 4.8 mm, OD 7.9 mm) to the Super Q filter. Following attachment of the N₂ and vacuum lines, the vials were placed into the Al block at 70°C until all solvent vaporized and moved through the system. At this time the dry vial was transferred to a second heating block at 200°C for 2 min. Blocks of Al (20 × 8 × 4 cm) were purchased from a local scrap metal dealer and holes were milled with a 31/64" bit to a depth of 3.25 cm resulting in near-perfect fit of the 4 ml glass vials. Heating mantle and Al block temperatures were monitored with oil immersed thermometers. Volatiles trapped by VPE were then eluted with 150 μl MeC₆H₄ and analysed by CI-GC/MS.

**Chemical ionization gas chromatography/mass spectrometry**

CI-GC/MS settings were previously described by Engelberth et al. (2003). Briefly, an HP 6890 gas chromatograph equipped with a split/splitless injector (splitless mode, 250°C, injection volume 1 μl) was interfaced to an HP 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) operated in chemical ionization (CI) mode with isobutane as the ionization gas. Compounds were separated on an HP-1MS (30 m × 0.25 mm × 0.25 μm) column held at 40°C for 1 min after injection, and then temperature programmed at 15°C min⁻¹ to 250°C (10 min), with helium as the carrier gas (0.7 ml min⁻¹). Specific CI conditions including ion source temperature, isobutane reaction gas pressure in the MS housing, filament emission current and electron energy were 250°C, 1.0 × 10⁻¹ torr, 100 μA, and 90 eV, respectively. Measurements were carried out either in total ion count (TIC) for pure standards or in SIM (selected ion ±0.7 mass units) for biologic samples. In Arabidopsis, 12 carboxylic acid ME analytes were measured using SIM with retention times and [M + H]⁺ ions as follows: (i) BA (7.38 min, 137), (ii) SA (8.44 min, 153), (iii) CA (10.10 min, 163), (iv) JA (trans 12.38 min/cis 12.61 min, 225), (v) IAA (13.47 min, 190), (vi)ABA (15.36 min, 279), (vii) linoleic acid (15.51 min, 295), (viii) linolenic acid (15.53 min, 293), (ix) oleic acid (15.56 min, 297), (x) steric acid (15.76 min, 299), (xi) OPDA (trans 17.08 min/cis 17.52 min, 307), and (xii) COR (19.55 min, 334). Internal standards with the corresponding methyl ester retention times and [M + H]⁺ ions are as follows: (i) cis-BA (7.38 min), (13C₂-BA (7.38 min, 143), (ii) 13C⁶-IAA (8.43 min, 157), (iii) 13C⁶-ABA (10.15 min, 168), (iv) JA (12.42 min/cis 12.66 min, 227), (v) 13C⁶-IAA (13.46 min, 195), (vi) 13C⁶-ABA (13.53 min, 285), and (vii) 13C⁶-linolenic acid (15.53 min, 311). The 13C⁶-IAA internal standard is converted to 13C⁶-SA-ME during the sample processing to give a 13C⁶-[M + H]⁺ ion instead of 159. The 279 and 285 parent [M + H]⁺ ions for ABA-ME and 13C⁶-ABA-ME fragment to produce predominantly 261 and 267 [M-H₂O + 1]⁺ ions, respectively, and were used for quantification purposes. Estimations of steric, oleic, linoleic and linolenic OPDA were based on 13C₁₈-linolenic acid. COR quantification utilized an external standard curve of COR-ME and previously determined recoveries (Schmelz et al., 2003).

Following TMS derivatization with BSTFA retention times and [M + H]⁺ ions were as follows: ACC (2 TMS-8.76 min, 246), SA (1 TMS-10.00 min, 211), JA (1 TMS-13.26 min, 283), IAA (2 TMS-14.64 min, 320), 2IP (1 TMS-15.89 min, 276), ABA (1 TMS-16.16 min, 319), linolenic acid (1 TMS-16.48 min, 351), OPDA (1 TMS-18.40 min/cis 18.92 min, 365), Z (1 TMS-19.94 min, 292), COR (1 TMS-20.29 min, 392), and GA₃ (2 TMS-22.00 min, 475). With the exception of GA₃, the [M + H]⁺ parent ions of the TMS derivatives predominated. The predicted 475 [M + H]⁺ ion for 2-TMS GA₃ displayed relatively low signal intensity relative to the abundant 341 ion fragment.

In general, isobutane CI is not as commonly used as methane CI and does not produce enough fragmentation ions for tuning purposes. Thus, while the low fragmentation generated by isobutane is a huge benefit for analytical sensitivity, the MS must first be tuned with methane. Switching between gases requires pumping out of the gas lines for at least 20 min, and then continuing to purge with the new gas on for an additional 10 min. Upon successful switching to isobutane CI the m/z 41 should be small compared with m/z 43. On the HP 5973 MS used in this work, isobutane flow is adjusted such that the m/z 47/43 ratio is in the 15–20 range (the limits are 5–30). Switching back to methane again requires rigorous purging and monitoring of the 41/43 ratio as an indication of how much isobutane remains. Methane autotune procedures will not work well in the presence of residual isobutane. To improve pumping capacity and instrument vacuum for continuous CI-MS operation, the original Agilent 5973 MS was modified by replacing the 37 l min⁻¹ roughing pump (Edwards E2M1.5; BOC Edwards, Wilmington, MA, USA) with a 117 l min⁻¹ (Edwards RV5) pump, and also replacing the 1/2" tubing between the roughing and turbo pump with 1" tubing. By design, CI requires routine maintenance and cleaning. Care must be taken to properly align the filament and to keep the emission voltages low to minimize the tendency of the filaments to expand and bend out of alignment that can cause a significant reduction in instrument sensitivity.

**Biotic stress experiments.** Arabidopsis thaliana Columbia (Col-0) were used to examine plant responses to Pseudomonas syringae pv. tomato DC3000 (PstDC3000). Both plant growth and pathogen infection have previously been described (O’Donnell et al., 2003). Twelve hours before infection plants were enclosed in a humidity chamber to aid bacterial entry. Five-week-old plants were inoculated with Pst DC3000 by submerging the whole plant in...
1 x 10^6 cfu ml^-1 bacterial suspension, containing MgCl2 (10 mm) and Silwet L-77 (0.02% v/v), for 30 sec and returned to the humidity chamber overnight. Mock-inoculated controls were treated similarly in the absence of Ps. Plant tissues were harvested, snap-frozen in liquid N2, pulverized, and stored at −80°C prior to analysis. Individual Ps. infected plants (n = 4) were successively harvested at 0, 24, 48, 72, and 96 h after treatments.

Recovery and breakthrough

To document the phenomenon of breakthrough, straight chain alkane mixtures, ranging between C7-C18 and C18-C38, were used to examine the relationship between analyte bp and recovery at two different flow rates for volatile collection. Temperature, collection times, and flow rates were 200°C, 2 min, 50 and 500 ml min^-1, respectively. Alkane mixtures were constructed at 1 µg of each analyte in a total volume of 100 µl MeCl2. For each flow rate and alkane series, recoveries were calculated based on GC-FID peak area of each analyte in unmanipulated standards (n = 4), normalized to 100%, and compared with the corresponding areas recovered by eluting analytes trapped by VPE (n = 4) and eluted with the original solvent volume (100 µl MeCl2).

To examine recovery of FA-MEs from a plant matrix, a series of 22 C8-C24 FA-MEs (1 µg of each in total volume of 150 µl MeCl2) were spiked into 200 mg of corn tissue (n = 4). Using the standard extraction and VPE protocol, without derivatization, recoveries were spiked into 200 mg of corn tissue in the absence of chamber overnight. Mock-inoculated controls were treated similarly with 100 µl MeCl2. To represent the effects of parameters varied, relative recoveries (n = 4) were determined by GC-FID response, with the largest mean peak area recovered for each analyte normalized to 100%. Actual recoveries of free acids and VOCs from plant tissue has previously been determined using a fixed protocol (Schmelz et al., 2003).

Analyte confirmation

Proportionally, phytohormones-MEs are often minor components of the ion chromatogram and, given this complexity, the identity of individual peaks needs to be confirmed within each plant species examined. Using previously reported GC conditions (Engelberth et al., 2003) structural confirmation of endogenous analytes with authentic standards was performed on selected biologic samples using a Trace GC 2000 coupled to GCQ iG- trap MS (Thermo Finnigan, San Jose, CA, USA) using MS/MS mode and methanol Cl with a source temperature of 200°C. The method was based on Muller et al. (2002) with modifications. All components of interest were analyzed using a scan time of 0.53 sec with 3 µs scans and max ionization time of 25 msec. Parent-ion selection was segmentally switched within each run with an isolation window of ±2 MU for each parent ion and with an isolation time of 8 msec. The collision energy was 2.0 V with a collision time of 30 msec and Q 0.450 V.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2167/TPJ2167sm.htm

**Figure S1.** Estimation of response linearity and detection limits of pure methyl ester (ME) standards analyzed by isobutane CI-GC/MS SIM at the levels of 10 pg, 100 pg and 1 ng (n = 3). Note: log/log scale.

(a) benzoic acid (BA-ME; r² = 0.998), (b) salicylic acid (SA-ME; r² = 0.992), (c) cinnamic acid (CA-ME; r² = 0.996), (d) jasmonic acid (JA-ME; r² = 0.990), (e) indole-3-acetic acid (IAA-ME; r² = 0.991), (f) abscisic acid (ABA-ME; r² = 0.996), (g) linolenic acid (18:3-ME; r² = 0.994), (h) 12-oxo-phytodienoic acid (OPDA-ME; r² = 0.992), and (i) coronatine (COR-ME; r² = 0.985). Selected ions monitored are given in the lower right-hand corner of each figure (a–i). Given the peak area and slope of the regression line, 1 pg of material will be below the detection limit for all compounds with the possible exception of (a) BA-ME and (d) JA-ME.

**Figure S2.** Arabidopsis tissue (g FW) requirement (mixture of mock-inoculated control and PsDC3000-infected plants) and detection limit for VPE isobutane CI-GC/MS SIM peak areas of selected analytes using 1, 10, and 100 mg leaf tissue. Note: log/log scale.

(a) benzoic acid (BA-ME; r² = 0.866), (b) salicylic acid (SA-ME; r² = 0.939), (c) cinnamic acid (CA-ME; r² = 0.975), (d) jasmonic acid (JA-ME; r² = 0.973), (e) indole-3-acetic acid (IAA-ME; 100 mg requirement), (f) abscisic acid (ABA-ME; r² = 0.970), (g) linolenic acid (18:3-ME; r² = 0.935), (h) 12-oxo-phytodienoic acid (OPDA-ME; r² = 0.964), and (i) coronatine (COR-ME; 10 mg requirement). Selected ions monitored are given in the lower right-hand corner of each figure (a–i). Circled regions represent three important deviations which include the following: (i) shallow BA-ME slope indicates background contamination with BA-ME, (ii) no detectable IAA-ME ions in the 1–10 mg tissue samples, (iii) no detectable COR-ME ions in the 1 mg tissue samples. Based on these results BA-ME levels cannot be accurately reported and the minimum tissue levels required for IAA and COR are 100 and 10 mg, respectively. Highly linear recovery from a plant matrix of all spiked free acid analytes as MEs (in the range of 60–1000 pg per injection) has previously been reported (Schmelz et al., 2003). Selected ions monitored are given in the lower right-hand corner of each figure (a–i).

Note: For the purposes of establishing variability and reproducibility, total selected ion peak area is plotted for the detection limits; thus, the variability between data points represents variation associated with the plant tissue aliquots, weighing and all steps of the VPE CI-GC/MS analysis. VPE conditions were 500 ml min^-1, at 200°C for 2 min.

**Figure S3.** Representative isobutane CI-GC/MS spectra of the predominant [M + H]+ parent ions of (a) SA-ME (rt = 8.44 min, m/z = 153), (b) CA-ME (rt = 10.10 min, m/z = 163), (c) JA-ME (rt = trans 12.38 min/cis 12.61 min, m/z = 225), (d) IAA-ME (rt = 13.47 min, m/z = 190), (e) ABA-ME (rt = 15.36 min,

m/z = 279), (f) 18:3-ME (rt = 15.53 min, m/z = 293), (g) OPDA-ME (rt = trans 17.08 min/cis 17.52 min, m/z = 307), and (h) COR-ME (rt = 19.55 min, m/z = 334). ABA-ME was the only analyte producing significant [M-H$_2$O + H]$^+$ fragmentation; thus, 261 was used for ABA-ME quantification purposes. Identical to the situation with (a) SA-ME and (f) 18:3-ME, BA-ME, linoleic acid-ME, oleic acid-ME, and steric acid-ME also produced predominantly [M + H]$^+$ parent ions with no significant fragmentation.

References


