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UHPLC–MS/MS based target profiling of stress-induced phytohormones

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ABSTRACT

Stress-induced changes in phytohormone metabolite profiles have rapid effects on plant metabolic activity and growth. The jasmonates (JAs) are a group of fatty acid-derived stress response regulators with roles in numerous developmental processes. To elucidate their dual regulatory effects, which overlap with those of other important defence-signalling plant hormones such as salicylic acid (SA), abscisic acid (ABA) and indole-3-acetic acid (IAA), we have developed a highly efficient single-step clean-up procedure for their enrichment from complex plant matrices that enables their sensitive quantitative analysis using hyphenated mass spectrometry technique. The rapid extraction of minute quantities of plant material (less than 20 mg fresh weight, FW) into cold 10% methanol followed by one-step reversed-phase polymer-based solid phase extraction significantly reduced matrix effects and increased the recovery of labile JA analytes. This extraction and purification protocol was paired with a highly sensitive and validated ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method and used to simultaneously profile sixteen stress-induced phytohormones in minute plant material samples, including endogenous JA, several of its biosynthetic precursors and derivatives, as well as SA, ABA and IAA.

1. Introduction

Plants have evolved a number of unique defense mechanisms to adapt to changing ambient conditions. This ability to alter their growth and development is regulated by signalling of several phytohormones. Although the individual roles of jasmonates (JAs), abscisic acid (ABA) and salicylic acid (SA) in plants' responses to various biotic and abiotic stresses are quite well established, stress-related JAs signalling in the modulation of numerous developmental processes remains to be clarified (Li et al., 2001; Linkies and Leubner-Metzger, 2012). The jasmonates are short-chain alkylcyclopentenone and alkylcyclopentanone carboxylates that are formed via the lipoxygenase pathway. The *de novo* synthesis of jasmonic acid (JA) and its subsequent metabolism are both crucial in controlling the level of the bioactive hormone (Wasternack and Kombrink, 2010). JA accumulates very rapidly in both local and distal sites of wounded model plant Arabidopsis leaf tissues: its level increases noticeably within around two minutes after injury (Glauser et al., 2009). Simultaneously, its volatile methyl ester (MeJA) is generated to enable the rapid transmission of JA signalling after its demethylation to free JA in target tissues (Stitz et al., 2011). Crucial is the conjugation of primarily synthesized (+)-7-*iso*-JA, the initial product of JA biosynthesis. This compound is conjugated with the amino acid isoleucine (Ile) to form the most active JA compound and the ligand of the JA-receptor, (+)-7-*iso*-JA-Ile, which plays a key role in JA signalling (Fonseca et al., 2009; Sheard et al., 2010). Other JA conjugates with primarily non-polar amino acids including leucine (Leu), valine (Val), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and methionine (Met) have also been detected in various plant species and may have roles that go beyond environmental stress responses (Knöfel and Sembdner, 1995; Tamogami and Kodama, 1997). For example, JA-Trp acts as a potential regulator of auxin homeostasis via an unknown mechanism during root growth (Staswick, 2009; Guttierrez et al., 2012). In addition, a wide range of JA metabolites with different physiological functions have been described, including 11-OH-JA, 12-OH-JA, and 12-glucosylated or sulfonylated JA derivatives (Wasternack and Hause, 2013). Finally, both the biosynthetic precursor of JA, *cis*-(+)-12-oxo-phytodienic acid (OPDA), and its 16-carbon homolog dinor-OPDA (dn-OPDA) have been also shown to accumulate in wounded leaves (Stintzi et al., 2001).

Modern target profiling analyses involve the simultaneous measurement of several phytohormonal classes in order to determine their individual significance and control mechanisms. Two main conventional hyphenated techniques, liquid chromatography–mass

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spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS), have been widely used to determine the levels of endogenous phytohormones and thereafter their involvement in plant stress signalling. The development of an effective extraction and sample enrichment protocols together with selective and sensitive single-run analytical methods has made it possible to quantify the levels of JA, ABA, SA, IAA, and their metabolites in diverse plant tissues (Durgbanshi et al., 2005; Forcat et al., 2008; Matsuura et al., 2009; Müller and Munné-Bosch, 2011; Balcke et al., 2012; Liu et al., 2012). When selecting extraction solvents for these signalling molecules, it is important to account for their weak acidity ($pK_a = 4–5$) and structural diversity. JAs and ABA are readily extracted using water-miscible organic solvents such as methanol, ethanol or acetone (Creelman and Mullet, 1995; Yoshihara et al., 1996; Durgbanshi et al., 2005; Balcke et al., 2012; Liu et al., 2012; Glauser et al., 2014). Isopropanol and methyl *tert*-butyl ether (MTBE) are also widely used for extraction in both targeted and non-targeted metabolomic studies aiming to investigate changes in phytohormonal profiles (Glauser et al., 2008; Stumpe et al., 2010; Müller and Munné-Bosch, 2011; Ternes et al., 2011). While organic solvents offer better analyte solubility, sample contamination with pigments and other interfering compounds can be minimized by extraction into a buffer of neutral or acidic pH such as phosphate-sodium buffer (Prinsen et al., 2000; Novák et al., 2012). The processes used for further sample enrichment should be designed to suit the final analysis. The target profiling of many metabolites from crude plant extracts may be hindered by signal suppression, due to strong matrix effects. These can be mitigated by using solid-phase extraction (SPE), which has been used for decades in bioanalysis and nowadays is more employed than non-selective liquid–liquid partitioning and subsequent filtration (Dobrev et al., 2005; Fan et al., 2011; Glauser and Wolfender, 2013). Several multistep SPE methods combining silica-based reversed-phase (RP) sorbents with long alkyl chains (C18) or polymer-based RP materials with ion-exchange properties (mixed-mode sorbents) have proven to be effective for purifying acidic plant hormones (Baldwin et al., 1997; Dobrev et al., 2005; Balcke et al., 2012; Jikumaru et al., 2013). Because many plant hormones of interest are present only at trace levels in tissue samples and are non-volatile (with the exception of the volatile MeJA), RP-based separations using capillary-LC or ultra-high performance liquid chromatography (UHPLC) systems interfaced with electrospray tandem mass spectrometry (ESI–MS/MS) have become popular for their analysis (Wilbert et al., 1998; Segarra et al., 2006; Forcat et al., 2008; Müller and Munné-Bosch, 2011; Balcke et al., 2012). Non-destructive LC methods are also preferred due to minimal and straightforward sample preparation without the time-consuming analyte derivatisation procedures required by GC–MS approaches (Mueller et al., 1993, 2006; Engelberth et al., 2003). Many publications have described simultaneous quantitative analyses of stress-induced phytohormones such as JA, SA, and ABA together with the other phytohormone classes (cytokinins, auxins and gibberellins) using highly selective MS monitoring of precursor-to-product ion transitions - MRM mode (Tamogami and Kodama, 1997; Wilbert et al., 1998; Balcke et al., 2012; Kojima and Sakakibara, 2012).

Here, we describe a novel sensitive and selective profiling method for analysing a broad range of jasmonates and other stress-induced phytohormones including SA, ABA, and IAA. The new methodology significantly reduces the impact of matrix effects by using optimized conditions for sample extraction and purification, and allows the determination of analytes with diverse physicochemical properties present in the plant tissue at very low levels. The combination of an effective SPE process using a polymeric reversed-phase sorbent with a sensitive UHPLC–ESI–MS/MS method enabled the exact quantification of sixteen compounds (13 JAs, SA, ABA, and IAA) in minute Arabidopsis leaf tissues. Our novel high-sensitive method provides detailed insights into the changes in phytohormone profiles that occur following wounding and will be applicable in studies of many stress-related responses and developmental processes involving these phytohormones.

2. Results and discussion

The methodology reported herein was primarily designed to establish a sensitive MS-based approach for the simultaneous profiling of stress-induced phytohormones including most of the JA metabolites (Fig. 1). The protocol was developed to enable the rapid and effective extraction of target compounds from a minimal quantity of plant material, with efficient isolation and effective enrichment together with highly selective and extremely sensitive analysis.

2.1. Optimization of extraction and purification protocols

In general, the main purpose of sample preparation procedures prior to an analyte detection using selected analytical method is to reduce sample complexity while maintaining high extraction efficiency of target compounds – in this case, low-abundance phytohormones. Depending on the chemical properties of the extraction solvent, crude plant extracts may contain large quantities of substances that will interfere with subsequent analyses (e.g., proteins, carbohydrates, pigments and lipids). To develop an improved extraction protocol, we optimized the method of sample processing (extraction and purification) and sample size in order to minimize analyte losses and reduce contamination with non-polar extractable substances. To begin with, the extraction efficiencies of four cold methanolic solvents (80% methanol, 50% methanol, 10% methanol, and 10% methanol acidified with 0.1% formic acid) were tested. Arabidopsis shoot extracts with a fresh weight of 20 mg each were prepared in quadruplicate for each extraction solution and spiked with a mixture of JAs standards of 20 pmol each with the exceptions of MeJA (50 pmol) and *trans*/*cis*-(+)-OPDA (100 pmol). After flow-through purification using an Oasis® HLB (HLB) cartridge, the total yields for the JAs were 25 ± 14%, 27 ± 12%, and 56 ± 22% for cold 80%, 50% and 10% methanolic solutions, respectively. When the experiment was repeated using an acidified solution of aqueous methanol (10%), the overall JAs recovery for the extraction and one-step SPE protocol was 53 ± 28%. Interestingly, the yield of acidic plant hormones was not affected by acidifying the extraction solution. In accordance with previously published data (Urbanová et al., 2013), the plant pigment content of the extracts increased rapidly with the methanol content of the extraction solution, yielding samples of insufficient purity for UHPLC–MS/MS analysis. Therefore, the optimized amount of sample and type of organic extraction solvent would reduce ion suppression or enhancement caused by the sample matrix and interferences from metabolites as well as sample throughput by increasing the sample preparation time (Novák et al., 2012). Consequently, all subsequent experiments were performed using 20 mg samples extracted with unacidified 10% methanol.

To maximize the sensitivity and selectivity of the final MS-based analysis, we sought to combine the efficient extraction protocol described above with a purification protocol that would afford high analyte recovery. We therefore decided to use a one-step purification protocol based on a HLB column washed with 10% methanol and eluted using 80% methanol (Fig. 2). The HLB sorbent is described as a macroporous copolymer [poly-(divinylbenzene-co-*N*-vinylpyrrolidone)] with both hydrophilic and lipophilic retention characteristics. This polymeric reversed-phase sorbent has been also preferred SPE material for one-step SPE of acidic plant