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Rapid extraction of abscisic acid and its metabolites for liquid chromatography–tandem mass spectrometry

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Abstract

We have described a simple, reliable and rapid method of extracting and partially purifying the phytohormone (+)-abscisic acid and its catabolites for liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) analysis. Lyophilized tissue samples were powdered by high-speed agitation with ceramic beads for 5 s. Metabolites were extracted from the tissue powder using acetone–water–acetic acid (80:19:1, v/v) with the addition of deuterated internal standards for quantification. Essentially all endogenous hormones were recovered by three successive tissue extractions. However we demonstrated that, with the use of internal standards, one extraction with vigorous vortexing was sufficient to obtain accurate results (recovery 65–90%). Solvents were optimized for partial purification of abscisic acid and related compounds by solid-phase extraction using Oasis HLB cartridges. The eluted metabolites were then analyzed by LC–MS–MS. To illustrate the applicability of these techniques, we analyzed the levels of abscisic acid and metabolites in seeds and valves of *Brassica napus* siliques at two stages of development. We detected abscisic acid, phaseic acid, 7′-hydroxyabscisic acid, dihydrophaseic acid and abscisic acid glucose ester. In both tissues, dihydrophaseic acid was the major accumulating product, reaching 97 300 pmol/g dry mass in valves at 24 days after anthesis. The amount of abscisic acid in seeds was high at 24 days after anthesis (23 300 pmol/g dry mass), but low in the other samples (292–447 pmol/g dry mass).

Keywords: *Brassica napus*; Plant materials; Plant hormones; Abscisic acid

1. Introduction

S-(+)-Abscisic acid (ABA) is a key plant hormone that regulates numerous aspects of plant growth and development including embryo maturation, seed dormancy, stress responses and stomatal aperture [1]. In plant cells, ABA is synthesized and degraded continually. ABA catabolism can occur by several routes involving oxidation, reduction or conjugation [2]. The major oxidative pathway is via the 8′-hydroxylation of ABA and is catalyzed by ABA 8′-hydroxylase [3]. The primary product, 8′-hydroxy-ABA, rapidly cyclizes to form (−)-phaseic acid (PA) which is usually reduced further to form dihydrophaseic acid (DPA). There is also an apparently minor oxidative pathway giving rise to (+)-7′-

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hydroxy-ABA (7′-OH-ABA) and a reductive pathway producing ABA 1′,4′-diol. ABA and its oxidized products can also be conjugated as esters, particularly glucose esters, and as glucosides [1,4]. The major glucose conjugate of ABA is ABA glucose ester (ABA-GE), which exhibits little or no biological activity but appears to be a transported form of ABA [5]. The relationship of these pathways to each other and to the effects of ABA remains unclear.

Continual synthesis, transportation (in and out) and degradation dynamically maintains ABA levels in plant cells. Thus measurements of extracted ABA only reflect the net effects of the above processes and provide little information on the rate of ABA synthesis and inactivation. With the increasing use of genomic and proteomic approaches in biological research involving high-throughput analyses of the proteome and transcriptome, there is a corresponding need for high-throughput, multi-target analyses of metabolites [6]. An important aspect of this is to obtain profiles of families of metabolically related, low abundance signaling molecules such as ABA and its metabolites. ABA metabolic profiles will help define the relative importance of competing pathways, conversion efficiencies within pathways and the relationship between ABA metabolism, environmental conditions and development.

Accurate measurement of ABA and its metabolites critically depends on sample preparation involving efficient extraction and partial purification. Aqueous methanol, aqueous acetone, aqueous ethanol, or mixtures of solvents have been used to extract ABA and its metabolites in plant samples [7]. The extracts have been purified by one or combinations of the following methods: phase partitioning, ion-exchange column chromatography, thin-layer chromatography, C_{18} packed columns, and high-performance liquid chromatography (HPLC) [8,9]. Recently, Dobrev and Kaminek [10] reported the use of Oasis MCX cartridges to separate cytokinins from indole acetic acid and ABA. Following partial purification, ABA and its metabolites have been analyzed by immunoassay, gas chromatography (GC), or GC–mass spectrometry (MS). When GC or GC–MS was employed, derivatization of ABA and its metabolites to more volatile methyl esters was required and ABA-GE was hydrolyzed to release free ABA. The extraction and analysis of ABA and its metabolites have been tedious and time-consuming processes. However, recent developments in analytical methodology allow a more rapid extraction protocol and a more precise and comprehensive analysis using LC–electrospray ionization (ESI) MS–MS.

LC–ESI-MS–MS is a combination of HPLC and ESI-MS–MS. ESI, in the negative ion mode, is an efficient ionization method for acidic organic compounds, which dissociate to form $[M\text{-}H]^-$ (precursor) ions. The precursor ions selected by the first mass analyzer undergo collision-induced dissociation to produce characteristic fragment (product) ions, which are detected by the second mass analyzer. These diagnostic precursor–product ion transitions are used to identify individual compounds. Use of internal standards and standard curves enable the amounts of individual compounds to be quantified. This method is specific and sensitive and does not require chemical derivatization of analytes.

In this paper, we develop rapid methods for sample preparation and partial purification that allow quantitative, simultaneous measurement of ABA and its metabolites in plant tissues using LC–ESI-MS–MS. To illustrate the utility of these techniques, we employ them to quantify ABA and its catabolites in *Brassica napus* siliques.

2. Experimental

2.1. Metabolites

The following chemicals were prepared as described previously: (+)-ABA and (−)-PA [11], *S*-(+)-[8′,8′,8′,9′,9′,9′-$^2H_6$]ABA (ABA-d_6) [12], (+)-7′-hydroxy ABA (7′-OH-ABA) [13], *S*-(+)-[8′,8′,8′-$^2H_3$]ABA (ABA-d_3) [14], [7′,7′,7′-$^2H_3$]PA (PA-d_3) (using the same method as described in Ref. [11]), DPA (according to the method of Zeevaart and Milborrow [15]) and ABA-GE [16]. Radioactive (+)-[3′,5′,5′,7′,7′,7′-$^3H_6$]ABA (^{3}H-ABA) was synthesized as described previously [11].

2.2. Plant tissues

Brassica napus cv. Quantum plants were grown in

a greenhouse under natural light supplemented with artificial light and heating to provide a 16-h light (25 °C): 8-h night (18 °C) cycle. The humidity was maintained at 45–65%. Only the primary and two secondary inflorescences were retained on each plant. Siliques were separated into seeds and valves and weighed. The separated tissues were then frozen in liquid nitrogen, lyophilized and subsequently stored at room temperature until extracted.

2.3. Sample extraction

The initial procedure involved preparing lyophilized plant tissues (0.02–0.1 g dry mass) which were crushed in liquid nitrogen using a mortar and a pestle and extracted with 3 ml of acetone–water–acetic acid (80:19:1, v/v). Internal standards of ABA-d_3 and PA-d_3 were added from concentrated stock solutions at the beginning of the extraction. The amounts of internal standard that were appropriate for accurate results varied and were determined by preliminary analyses. The extracts were transferred to two 2-ml tubes and after centrifugation (Biofuge Pico, Heraeus Instruments, Germany) at 13 000 rpm for 2 min, the supernatant was collected and the residues were re-extracted with 3 ml extraction solvent. The second extract was centrifuged and then supernatants were combined and dried under a stream of nitrogen.

In later experiments freeze–dried tissue was chopped and placed in a 2-ml screw-cap microcentrifuge tube (Sarstedt, Montreal, Canada; Model 72.694.006) with a 1/4 in. (1 in.=2.54 cm) ceramic bead (Q-biogene, Carlsbad, CA, USA; Model 6540-424). The tissue was powdered by 5 s agitation using a Savant Fast Prep Model FP 120 (Q-biogene) to produce high-speed (6.5 m/s) random-motion shaking. If plant material weighed less than 50 mg, a lower speed (4.5 m/s) was used to reduce the likelihood of tube damage. Powdered tissue was extracted with acetone–water–acetic acid (80:19:1, v/v) with vortexing (1.2 ml for the first extraction and 1 ml each for the second and third extractions). Internal standards were added in the first extraction. To suspend valve tissues in the first extraction, the Savant FP 120 was used again for 2 s at a lower speed (4.5 m/s). After centrifugation, the supernatant of the first extract was transferred to a fresh tube and dried by centrifugation under vacuum (Vacufuge, Brinkmann Instruments, Mississauga, Canada) at 30 °C. Then the second supernatant was transferred to the tube containing the dried first supernatant and also dried as above. The same procedure was repeated for the third supernatant.

2.4. Purification by Oasis HLB cartridges

The dried extracts were dissolved in 100 μl of methanol–acetic acid (99:1, v/v) and then mixed with 900 μl of 1% acetic acid. The samples were clarified by centrifugation at 13 000 rpm for 1 min or filtered through a syringe filter tip before purification with 1 ml Oasis HLB cartridges (Waters, Mississauga, Canada). The sorbent of these columns (30 mg) is a co-polymer made from two monomers, divinylbenzene and *N*-vinylpyrrolidone.

Oasis HLB extraction cartridges were placed either on a vacuum manifold or in 14-ml polypropylene round-bottom tubes (Becton Dickinson Labware). The solutions were drawn through either by gentle vacuum (at no less than 75 000 Pa) or by centrifugation (IEC Centra CL2, International Equipment Company, MA, USA) with a swing bucket rotor at 1000 rpm for 1 min. Flow-rates were less than 1 ml/min. The cartridges were conditioned with 1 ml methanol and equilibrated with 1 ml methanol–water–acetic acid (10:89:1, v/v). The samples (about 1 ml in volume) were loaded onto the Oasis cartridges and washed with 1 ml methanol–water–acetic acid (10:89:1, v/v). Then ABA and metabolites were eluted by 1 ml methanol–water–acetic acid (80:19:1, v/v) and collected in (or transferred from a 14-ml tube to) a 2-ml flat-bottom eppendorf tube. The eluate was dried by centrifugation under vacuum at 30 °C.

2.5. LC–ESI-MS–MS procedure

The dried sample was reconstituted in 200 μl acetonitrile–water (15:85, v/v) containing 12 m*M* acetic acid (pH 3.3). A portion (1–10 μl) of the sample was loaded onto a HP1100 Series HPLC system (Hewlett-Packard) equipped with a 100×2.1 mm, 5 μm SB-C_{18} LC–MS column (a Zorbax column, Agilent Technologies Canada, Mississauga, Canada) using a flow-rate of 0.2 ml/min and a

binary solvent system comprising 12 m*M* acetic acid in water (A) and 12 m*M* acetic acid in acetonitrile–water (90:10, v/v) (B). Typically, the solvent gradient was programmed to change linearly from 15% B to 33% B over the first 10 min and then to 100% B over the next 6.7 min before returning to the initial composition at 22 min. The retention times were 5.4 min for DPA, 8.4 min for ABA-GE, 9.5 min for PA, 10.5 min for 7′-OH-ABA and 13.5 min for ABA. Analysis was performed with a Quattro LC instrument equipped with an electrospray (Z-spray) ion source and MassLynx version 3.5 software (Micromass, Manchester, UK). Specifically, the compounds were ionized by passing the LC eluent through a stainless steel electrospray capillary held at a potential of 2.75 kV. The solvent was removed with a 400 l/h flow of nitrogen gas heated to 250 °C. Precursor ions were extracted into the mass spectrometer through a sampling cone held at 25 V. Each analyte was scanned to confirm its mass-to-charge ratio (m/z) and retention time. The first mass analyzer was then programmed to select each precursor ion as it eluted from the LC column. Product ions produced by collision-induced dissociation of each precursor were detected by the second mass analyzer. The most abundant product ion was usually selected for multiple reaction monitoring (MRM), during which the response for each precursor–product ion transition was used to quantify the corresponding amount of ABA and its metabolites.

Endogenous ABA and ABA-GE was quantified by the comparison of their peak areas to that of ABA-d_3. Endogenous PA, DPA and 7′-OH-ABA were quantified by the comparison of their peak areas to that of PA-d_3. Detailed LC–ESI-MS–MS procedures for analysis of ABA [17] and ABA metabolites will be described elsewhere.

Typically, for a 10-μl injection of sample prepared with 20 ng internal standard and reconstituted in 200 μl initial mobile phase, the limit of detection (LOD) and limit of quantification (LOQ) were calculated from calibration curves and samples using the Quantify module of MassLynx version 3.5 software. The respective LODs and LOQs are 0.02 and 0.06 ng/100 mg dry mass for ABA, 0.27 and 0.73 ng/100 mg dry mass for ABA-GE, 0.05 and 0.13 ng/100 mg dry mass for PA, 0.07 and 0.18 ng/100 mg dry mass for DPA.

3. Results

3.1. Hydrolysis of ABA-GE in alkali solution at room temperature

Previous investigations have shown that ABA-GE was hydrolyzed in strongly alkaline solutions at 60 °C [18,19], but it was not clear how susceptible ABA-GE was to hydrolysis under more moderate conditions. To test this, ABA-GE was incubated in various basic and acidic solutions at 23 and 60 °C. ABA-GE was completely hydrolyzed by 0.1 *M* NaOH at 60 °C in 3 min and at 23 °C in 30 min (Fig. 1). Even 0.01 *M* NaOH hydrolyzed 70% of ABA-GE in 60 min at 23 °C. However, at room temperature, ABA-GE was stable in water, 1% acetic acid or 10% acetic acid.

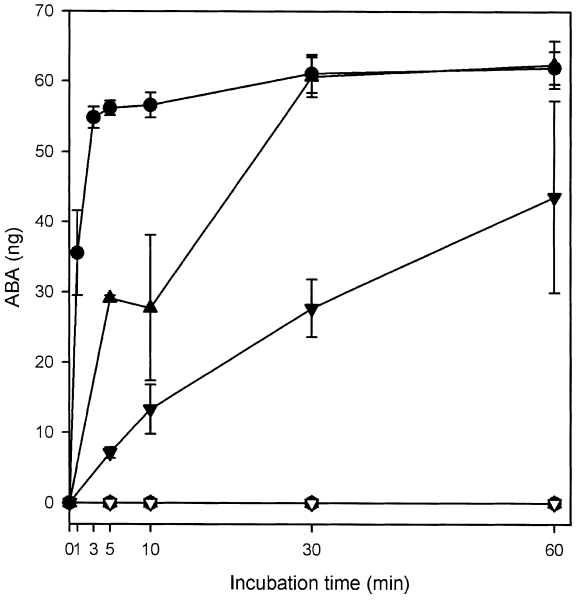

Fig. 1. Hydrolysis of ABA-GE to release ABA. ABA-GE (aliquot of 2 μg) was dissolved in 1 ml of water (hollow circle), 0.01 *M* NaOH (filled triangle down), 0.1 *M* NaOH (filled triangle up), 1% acetic acid (hollow triangle up) or 10% acetic acid (hollow triangle down) and incubated at 23 °C. ABA-GE (2 μg) in 0.1 *M* NaOH (solid circle) at 60 °C was used as a positive control (complete hydrolysis). Aliquots of 50 μl taken at appropriate times were acidified immediately by mixing with 50 μl of 10% acetic acid and 80 ng ABA-d_3. ABA was analyzed by LC–MS–MS and amounts calculated by reference to ABA-d_3 as an internal standard. Values are means±SE of three replicate treatments.

3.2. Optimal methanol concentration for sample loading

Since ABA is not readily soluble in acidified water while alkaline conditions cause ABA-GE hydrolysis, we used acidified (1% acetic acid), aqueous methanol as a general solvent. To determine the methanol concentration for loading of sample extract on to Oasis HLB cartridges, we tested a series of methanol concentrations (Fig. 2). For ABA, a solution containing 30% methanol was the highest concentration that allowed for cartridge equilibration, loading and

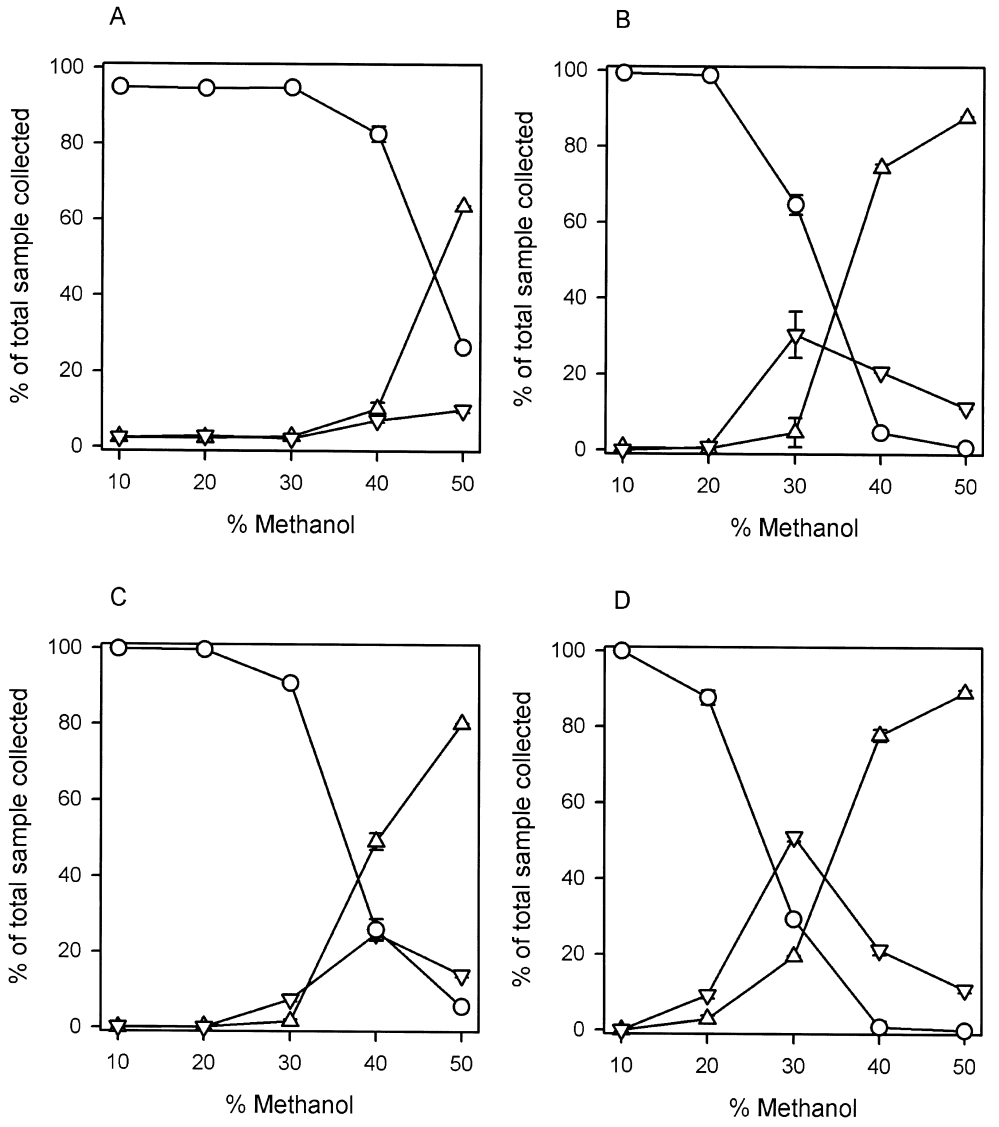

Fig. 2. Effects of methanol concentration on ability of Oasis 1 ml HLB cartridges to retain ABA, ABA-GE, PA and DPA. A mixture of (A) ABA, (B) ABA-GE, (C) PA and (D) DPA (20 ng each) were added to 1 ml 10, 20, 30 or 40% methanol containing 1% acetic acid and loaded to a cartridge. The acidified methanol concentration for equilibration and washing (1 ml volume) was the same as that for sample loading. The analytes were eluted by 1 ml methanol. Then 20 ng ABA-d_3 and PA-d_3 were added to the void volumes and methanol eluates for LC–MS–MS analysis. The void volumes (hollow triangle up), washes (hollow triangle down) and methanol eluates (hollow circle) were monitored for ABA (A), ABA-GE (B), PA (C) and DPA (D). Values reported are means±SE of three replicates.

washing with complete retention of ABA on the cartridge. For ABA-GE and PA, the maximum methanol concentration was 20% while 10% methanol was the maximum concentration for DPA. Although we did not include 7′-OH-ABA in these experiments (due to insufficient material), subsequently we observed that the chromatographic behavior of 7′-OH-ABA was intermediate between that of ABA and PA (data not shown), therefore 10% methanol was used as a general concentration for sample loading.

3.3. Optimal methanol and acetonitrile concentration to elute ABA and metabolites

Next we determined the acidified (1% acetic acid) methanol concentration required to completely elute all of the compounds from Oasis HLB cartridges (Fig. 3A). We found that 50% methanol was sufficient to completely elute ABA-GE and DPA while 70% methanol was required for complete elution of PA and 80% was required for complete elution of ABA. Subsequently, we observed that 7′-OH-ABA was eluted by the same methanol concentration as PA (data not shown). Therefore, 80% methanol was used to elute ABA, ABA-GE, PA, DPA and 7′-OH-ABA.

We also found that 20% acetonitrile eluted most of DPA, ABA-GE and PA and 10–15% of ABA. All ABA was eluted by a concentration of 40% acetonitrile. Therefore, 40% acetonitrile could also be used to elute ABA and metabolites (Fig. 3B).

3.4. Efficiency of extraction

To test the efficiency of our extraction method, we used seeds and valves from three single *Brassica napus* cv. Quantum siliques at different stages of

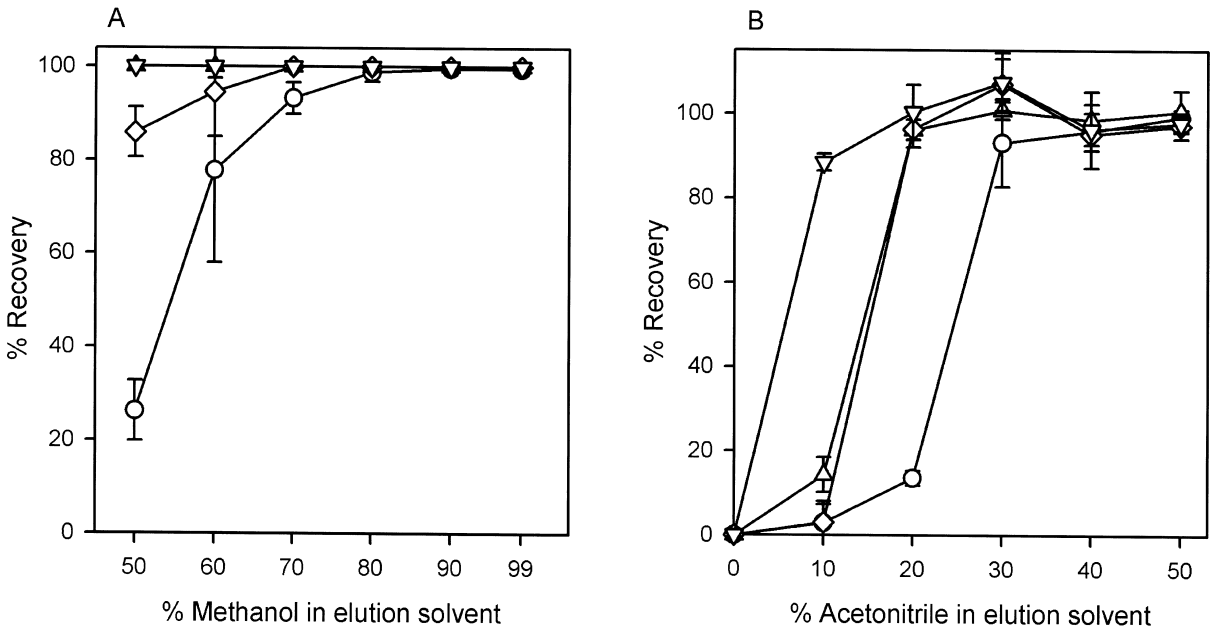

Fig. 3. Effects of methanol and acetonitrile concentration on elution of analytes from 1 ml HLB cartridges. A mixture of ABA (hollow circle), ABA-GE (hollow triangle up), PA (hollow diamond) and DPA (hollow triangle down) (20 ng each for A and 40 ng each for B) was loaded to a cartridge equilibrated with 1 ml methanol–water–acetic acid (10:89:1, v/v). (A) Effects of methanol concentration. The cartridge was washed with 1 ml methanol–water–acetic acid (10:89:1, v/v) and eluted with 1 ml acidified 50, 60, 70, 80, 90 or 99% methanol. Then 20 ng ABA-d_3 and PA-d_3 each were added as internal standards to the eluted analytes for LC–MS–MS analysis. (B) Effects of acetonitrile concentration. The cartridge was washed with 1 ml 1% acetic acid and eluted with 1 ml acidified 10, 20, 30, 40 or 50% acetonitrile. Then 40 ng ABA-d_3 and PA-d_3 each were added as internal standards to the eluted analytes for LC–MS–MS analysis. Values are means ± SE of three replicates.

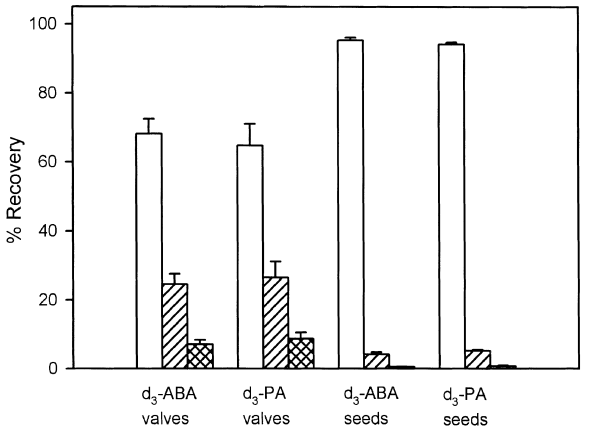

Fig. 4. Efficiency of the high throughput extraction. The valves and seeds of three *Brassica napus* siliques were powdered and extracted by the method described in the Experimental section. ABA-d_3 and PA-d_3 (20 ng each for valve samples and 200 ng ABA-d_3 and 100 ng PA-d_3 for seed samples) were added to a sample in the first extraction. The first (no fill), second (diagonal lines) and third extracts (crosshatch) were mixed with ABA-d_6 (internal standard for quantifying recovery) and purified for LC–MS–MS analysis. The efficiency of individual extractions was calculated by assuming 100% recovery of d_3-analytes from three successive extractions. Values are means±SE of three replicates.

development as test materials (Fig. 4). The first extraction of the valves recovered 65–70% of both d_3-internal standards whereas for seeds the corresponding recovery was better than 90%. The second extraction recovered 25% of the valve internal standards and 3–4% of the seed internal standards. The third extraction recovered about 10% of the valve internal standards and 1% of the seed internal standards.

However, with the use of internal standards, it is likely that calculated concentrations based on one extraction will be almost identical to the concentration calculated from three consecutive, combined extractions. For this to be true, both the internal standard and endogenous analytes must reach equilibrium during the extraction between the solid and liquid phases. To test this, we compared apparent concentrations based on recoveries from one extraction with those calculated from samples containing three extractions combined (Table 1). This data confirms that several extractions are required to approach complete (100%) analyte extraction. The results also show that vigorous agitation improves extraction and that, providing the first extraction is accompanied by vortexing, one extraction is sufficient to provide an accurate measure of concentration.

3.5. Levels of ABA and its metabolites in Brassica napus siliques

Table 2 shows the amounts of ABA and its metabolites in *Brassica napus* siliques that have been separated into seeds and valves at two development stages: 24 days after anthesis (seed balloon stage) and 44 days after anthesis (seed physiological maturity stage). At 24 days after anthesis, the ABA level in seeds was 23 280 pmol/g dry mass while

Table 1
Effects of shaking or vortexing during extraction on the apparent ABA levels in *Brassica napus* tissues (ng/sample)

Extract	Shaking						Vortexing					
	Silique 1		Silique 2		Silique 3		Silique 4		Silique 5		Silique 6	
	Valve	Seed	Valve	Seed	Valve	Seed	Valve	Seed	Valve	Seed	Valve	Seed
First	137.8	21.3	93.4	7.1	45.8	1.7	283.4	22.6	111.3	31.4	383.1	2.52
First+second	211.8	52.6	112.0	9.1	62.8	2.0	313.3	23.3	119.3	32.5	422.1	1.97
First+second+third	239.6	62.7	119.1	9.8	65.8	2.0	324.9	23.5	122.6	33.1	440.1	2.58

Six individual siliques of various ages were analyzed. To each silique, ABA-d_3 was added before processing (20 ng to each silique valve sample and 200 ng to each seed sample). Powdered tissues were first extracted with 1.2 ml acetone–water–acetic acid (80:19:1, v/v) by either shaking or vigorous vortexing. Shaking was accomplished with an Thermomixer 5436 Eppendorf at a setting of 800 rpm for 5 min. Vortexing was performed at maximum setting for 1 min, ensuring complete tissue suspension. The residues were extracted a second and third time with 1 ml acetone–water–acetic acid (80:19:1, v/v) and vortexing. Then ABA-d_6 was added to each extract as a recovery standard. The amount of endogenous ABA and ABA-d_3 in each extract was calculated by the comparison of their peak areas with that of ABA-d_6. The quantity of endogenous ABA was calculated as follows: the apparent amount of endogenous ABA multiplied by the actual amount of added ABA-d_3 divided by the amount of ABA-d_3 measured by MS–MS.

Table 2
Levels of ABA and its metabolites in *Brassica napus* siliques

Sample	ABA	ABA-GE	PA	DPA	7′-HOABA
Seeds at ca. 24 DAA	23 300±530	229±7	654±60	27 200±1200	1556±42
Valves at ca. 24 DAA	376±50	126±62	25±9	97 300±4700	809±94
Seeds at ca. 44 DAA	292±22	233±12	–*	–*	208±43
Valves at ca. 44 DAA	447±50	298±65	7.0±2.4	35 900±3000	1261±139

The levels of metabolites are expressed as pmol/g dry mass except those of 7′-OH-ABA which are expressed as relative response to standard ABA. Data shown are means±SE of 14 siliques for samples from ca. 44 days after anthesis (abbreviated to DAA in the table) and five siliques for samples from ca. 24 days after anthesis. *: ''–'' indicates that only one sample contained a trace of the analyzed compound.

valve ABA content was at a relatively low level of only 376 pmol/g dry mass. Meanwhile, seeds also contained much higher levels of ABA-GE, PA and 7′-OH-ABA than in valves. However, DPA levels in valves was 97 300 pmol/g dry mass, about 3–4-fold greater than in seeds. At 44 days after anthesis, ABA contents in both seeds and valves were at basal levels. At this time, seed DPA and PA levels had decreased to undetectable levels while valve DPA levels had fallen to 36 000 pmol/g dry mass. At 44 days after anthesis, seed 7′-OH-ABA level was only 1/6 of that in valves.

4. Discussion

4.1. Tissue extraction

As noted in the Introduction, ABA is readily soluble in a variety of solvents [7,20]. The most common solvents used for extractions from plant tissue have been acidified (0.5–2%) acetone (70–100%) or aqueous methanol (80–90%) [7,18–20]. These solvents are also appropriate for the extraction of PA, DPA and ABA-GE. Some authors recommend the addition of antioxidants to prevent ABA oxidation during the extraction [15,18,21] but the ability of these compounds to prevent the breakdown of ABA has not been proven [20]. Milborrow and Mallaby [18] indicated that alkaline methanol or even neutral methanol could react with ABA-GE to form ABA-methyl ester (ABA-Me) and it has been suggested that the formation of ABA-Me might result from the breakdown of bound ABA, of unknown structure [22,23]. To avoid the formation of ABA-Me, we used acetone–water–acetic acid (80:19:1, v/v) as the extraction solvent [19]. However methanol–water–acetic acid (80:19:1, v/v) also gave consistent results.

The rate-limiting step in preparing extracts was initially tissue homogenization using a mortar and pestle. To accelerate the extraction, we modified the procedure by powdering dried tissue with ceramic beads. This method is much faster than the mortar and pestle for homogenizing tissue and has reduced chances of sample loss and cross-contamination.

Our results on extraction efficiency showed that two extractions are enough to obtain about 90% or better recovery for added internal standards (Fig. 4) and endogenous ABA, ABA-GE, PA and DPA (data not shown) for seeds and valves. The first extraction only yielded about 65–70% for valves, recoveries much lower than from seeds. This likely resulted from the larger valve mass, which retained more of the extract.

In this paper we used three extractions to maximize recovery in order to ensure that analytes were clearly above the sensitivity limits of MS–MS. In some cases it may be desirable to simplify the procedure by employing only one extraction. For accurate quantitation of hormone levels it is important to ensure that the apparent amounts of analytes (and therefore the ratios of endogenous analytes to internal standards) are consistent in successive extractions. The results in Table 1 show consistent estimates of concentration, providing that agitation by vortexing is employed. Therefore, reliable values for amounts of analytes could be obtained from only one extraction. This may be useful for situations in which a simplified procedure (e.g., for large sample numbers, possibly employing robotic technology) is required.

4.2. The stability of ABA-GE

ABA-GE has been identified as a significant ABA catabolite for many years [4]. However, interest in this compound has increased substantially due to recent reports that it is a transported form of ABA [5]. Therefore, we examined the stability of ABA-GE under acid, neutral and basic conditions to ensure that we were able to obtain accurate quantitation of this compound. Since ABA, PA and DPA are weak acids (for ABA pK=4.7), their solubility is maximal at alkaline pH. Unfortunately, we have shown that ABA-GE is extremely labile at alkaline pH and previous results suggest that PA-GE is likely to be similarly sensitive [24]. However, ABA-GE was stable under acidic conditions. These results necessitated the use of acidified water or organic solvents to solubilize and partially purify ABA and its metabolites.

4.3. The behavior of ABA and its metabolites on chromatography

The behavior of ABA, *epi*-DPA, PA and DPA on reversed-phase HPLC has been reported previously [25,26]. Usually, solutions containing these analytes were acidified to suppress ionization of acids before being loaded on to the HPLC system. The polar compounds (DPA and PA) eluted earlier than less polar compounds (ABA). Before the samples were loaded onto the HPLC system, they were often purified with a C_{18} cartridge to remove lipophilic pigments. In this paper we used Waters Oasis HLB extraction cartridges to purify the ABA and its metabolites prior to LC–MS–MS. The sorbent of this cartridge is a copolymer made from two monomers, the lipophilic divinylbenzene and hydrophilic *N*-vinylpyrrolidone. It has higher capacity than silica-based C_{18} but retains polar compounds better than C_{18}-bonded silica solid-phase extraction (SPE) adsorbents. Currently, it is mainly used in SPE of body fluids. We showed that the chromatographic behavior of ABA and its metabolites on Oasis HLB was similar to elution from a reversed-phase column, i.e., DPA first, then PA, and finally ABA. This similarity helps us to predict the behavior of other likely metabolites on the cartridges.

We found that the capacity of the 1 ml Oasis HLB cartridge for ABA was 2.4 mg (data not shown). This is consistent with the manufacturer's claim that the maximum capacity of a 1 ml Oasis HLB cartridge (which contains 30 mg sorbent) is 1–5 mg. Tissue samples used in this report contained approximately 100 mg of dry mass. We found that acetone extracts (combined from three successive extractions) from seeds and valves contained about 10 mg dry mass. When the extracts were loaded to Oasis HLB cartridges, the majority of the dry mass either flowed through the columns or was eluted with the 1% acetic acid wash. When we fed ^{3}H-ABA (1 μg; 2×10^{6} dpm/silique) to the surface of siliques (seed pods), the harvested valves (90 to 155 mg dry mass) were extracted and loaded to the cartridges. We detected less than 5% radioactivity in the void volume plus wash fraction for all nine tested samples. Therefore, when the tissue mass extracted was in the range of 100–150 mg dry mass, the amounts of analytes were well within the capacity of the columns.

The use of solid-phase extraction avoids the use of time-consuming solvent partitioning. Partial purification using Oasis HLB columns is rapid and numerous samples can be processed in parallel. At present, in less than 1 h, we can process 20 samples using a vacuum manifold or 16 samples by centrifugation. This purification method may also be used prior to immunoassay (enzyme-linked immunosorbent assay, ELISA), GC or GC–MS analysis for ABA and its metabolites with no modifications. The Oasis HLB cartridge could be adapted for the purification of very polar ABA metabolites (such as DPA-glucoside or DPA-glucose ester) and other plant hormones such as gibberellins and indole acetic acid. For example, we found that acetonitrile–water–acetic acid (50:49:1, v/v) was sufficient to elute indole acetic acid from the cartridges (data not shown).

4.4. ABA metabolism during Brassica silique development

To illustrate the utility of the combined extraction, purification and LC–MS–MS methods described above, we determined levels of ABA and its metabolites in valves and seeds of single siliques from individual plants. This is the first study in which levels of both ABA and its metabolites were ana-

lyzed in canola siliques. ABA plays a key role in seed development but the contribution of the various ABA catabolic pathways to regulating ABA levels has not been studied. In addition, the relationship and possible interaction between ABA metabolism in seed and in the valves (which provides nutrients to the seed via the replum) is unknown. The presence of ABA metabolites such as PA, DPA, ABA-GE, and 7′-OH-ABA in both seeds and valves showing that catabolism by 7′- and 8′-hydroxylation, as well as glucose conjugation, was occurring. Seeds at 24 days after anthesis contain high levels of DPA suggesting that 8′-hydroxylation was likely the primary route of ABA catabolism.

Results presented in Table 2 indicated that at 24 days after anthesis, seed ABA levels were about 23 000 pmol/g dry mass, about 60-fold higher than in valves. At 44 days after anthesis, seed ABA levels had decreased to 292 pmol/g dry mass, less than 2% of the amount at 24 days after anthesis, while valve ABA levels slightly increased (by 53%). The large difference between seed ABA levels at 24 days after anthesis and 44 days after anthesis suggests that ABA catabolism is very active before seed physiological maturity.

At 24 days after anthesis valve DPA levels were fourfold higher than seed ABA and threefold higher than seed DPA. There appears to be active ABA catabolism in valves such that ABA levels are maintained relatively low. The decrease of DPA in both seeds and valves at 44 days after anthesis suggest that DPA is further metabolized. The further catabolism of PA and DPA is not unexpected and has been observed previously [27].

In this paper, we described the development of a rapid extraction and purification method for metabolic profiling of ABA and its metabolites by LC–MS–MS. Using these techniques we measured the levels of ABA and metabolites in seeds and valves of single *Brassica napus* siliques. Our results showed that the main pathway of ABA catabolism is via 8′-hydroxylation to yield DPA but that ABA levels were regulated completely differently in seeds and valves. We also show that other catabolic pathways were detectable and may play a significant role in some circumstances. Further detailed study on ABA metabolism is needed to understand the role of the various ABA catabolic pathways in silique growth and development and the interaction between valves and seeds.

The methods described here will be applicable, with minimal modifications, to most other plant systems and will allow more detailed analysis of ABA metabolism in relation to plant growth and development.

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