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A New Abscisic Acid Conjugate, ABA-L-Glutamate, Determined in Different Plant Species by Combined Immunoaffinity Chromatography-Tandem Mass Spectrometry

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Abstract
Abscisic acid (ABA) is a phytohormone that occurs in plants at very low concentration (pmol/g fresh weight) and regulates multiple biological processes, including stomatal closure, seed germination, and responses to environmental stresses. In the present study, isolation of ABA, ABA glucosyl ester, and 11 ABA amino acid conjugates from minute quantities of plant tissue (less than 20 mg fresh weight) was achieved using a purification method based on the combination of an Oasis HLB column and an immunoaffinity sorbent. New monoclonal antibodies raised against (+)-*cis,trans*-ABA conjugated to BSA through its carboxyl group (C1) were characterised by enzyme-linked immunosorbent assay (ELISA) and used for immunoaffinity chromatography (IAC) gel preparation. The use of immunoaffinity purification significantly reduced matrix effects and increased the selectivity and sensitivity of subsequent UHPLC-MS/MS analysis. In addition to (+)-*cis,trans*-ABA and its glucosyl ester, a new abscisic acid conjugate, ABA-L-glutamate, was isolated by IAC and identified by tandem mass spectrometry in pea (*Pisum sativum* L.), *Lepidium sativum* L. and wheat (*Triticum aestivum* L.) seedlings. However, it was not found in 10-day-old seedlings of *Arabidopsis thaliana* or water-stressed tobacco (*Nicotiana tabacum* L.) leaves. Here, the identification of an ABA conjugate with glutamic acid in plants is described for the first time.

Keywords Abscisic acid amino acid conjugate (ABA-AA) · Enzyme-linked immunosorbent assay (ELISA) · Monoclonal antibody (mAb) · Immunoaffinity chromatography (IAC) · Ultra-high-performance liquid chromatography (UHPLC) · Tandem mass spectrometry (MS/MS)

Introduction

The phytohormone abscisic acid (ABA) plays a central role in adaptive responses to abiotic and biotic stresses, e.g. drought, salinity, low temperature, wounding and pathogen attack (de Torres-Zabala et al. 2007; Zeevaart and Creelman 1988; Zhang et al. 2021; Zhu 2002), as well as regulation of a variety of plant developmental processes, including seed maturation, dormancy and germination, leaf senescence, primary root growth and flowering time (Chen et al. 2020).

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ABA is a sesquiterpenoid that is synthetised in the plastids of higher plants by an "indirect" pathway via cleavage of a C_{40} precursor β-carotene (Nambara and Marion-Poll 2005). The first step in the biosynthetic pathway is the epoxidation of zeaxanthin, which is converted by zeaxanthin epoxidase (ZEP) into violaxanthin via antheraxanthin and then to neoxanthin. Plants only utilise the 9-*cis* isoforms (9-*cis*-violaxanthin and 9′-*cis*-neoxanthin), which are converted to xanthoxin (C_{15}) via oxidative cleavage catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Nambara and Marion-Poll 2005). Xanthoxin is then exported from plastids to the cytosol, where it is converted to ABA by two oxidative reactions catalysed by xanthoxin dehydrogenase and aldehyde oxidase (Nambara 2017). It has been shown that β-apo-11-carotenoids (C15 apocarotenoids) derived from the cleavage of β-carotene and zeaxanthin are involved in a ZEP-independent ABA biosynthesis pathway (Jia et al. 2022). ABA catabolism occurs mainly through hydroxylation and conjugation (Fig. 1). The 8´-hydroxylation pathway is the main route and produces an unstable intermediate, 8´-OH-ABA, which is spontaneously converted into the more stable phaseic acid (PA). This reaction is catalysed by the CYP707A family of cytochrome P450s (Kushiro et al. 2004; Saito et al. 2004). PA is further modified by PA reductases, converting it to dihydrophaseic acid (DPA) and *epi*-dihydrophaseic acid (*epi*-DPA). The 7´- and 9´-hydroxylation pathway produces 7´-OH-ABA and 9´-OH-ABA, respectively. 9´-OH-ABA is cyclised to neophaseic acid (neoPA) (Zhou et al. 2004), which is further converted by neoPA reductase 1 (NeoPAR1) into *epi*-neodihydrophaseic acid (*epi*-neoDPA) (Bai et al. 2022). However, the enzyme responsible for 7´-hydroxylation is still unknown. The reversible conjugation of ABA with glucose by glucosyltransferases (GTs) forms the ABA glucosyl ester (ABA-GE), which may be hydrolysed to free ABA by β-glucosidases (BG) (Dietz et al. 2000; Lee et al. 2006; Nambara and Marion-Poll 2005; Zaharia et al. 2005).

Acidic phytohormones such as indole-3-acetic acid (IAA), jasmonic acid (JA) and salicylic acid (SA) have been shown to conjugate to amino acids (Hladík et al. 2023; Ludwig-Müller 2011; Mík et al. 2023; Piotrowska and Bajguz 2011; Široká et al. 2022). Conjugates of JA with amino acids are bioactive components of plant tissues and may have roles other than responses to environmental stress. The main bioactive JA amino acid conjugate in plants is (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), which controls responses of vascular plants to herbivores as well as other JA-regulated processes (Fu et al. 2022; Piotrowska and Bajguz 2011; Wasternack and Hause 2013). Recent research suggests that conjugation of IAA to amino acids could play important roles in specific developmental processes (di Mambro et al. 2017; Zheng et al. 2016), whereas SA-amino acid conjugation is probably involved in SA catabolism. Salicyloyl-aspartate (SA-Asp), the only reported endogenous SA-amino acid conjugate in plants, has been shown to induce pathogenesis-related (PR) gene expression and increase disease resistance to pathogenic *Pseudomonas syringae* (Chen et al. 2013; Lefevere et al. 2020). However, amino acid conjugates of the other acidic plant hormones, gibberellins (GAs) and ABA, have not yet been reported in plants. Todoroki et al. (2011) prepared 19 ABA amino acid conjugates and investigated their biological activity, enzymatic hydrolysis by recombinant *Arabidopsis* amidohydrolases and metabolic fate in rice seedlings but did not determine their occurrence in plants.

Due to the very low concentrations (pmol g^{-1} fresh weight) of ABA and its metabolites in plant tissues, their

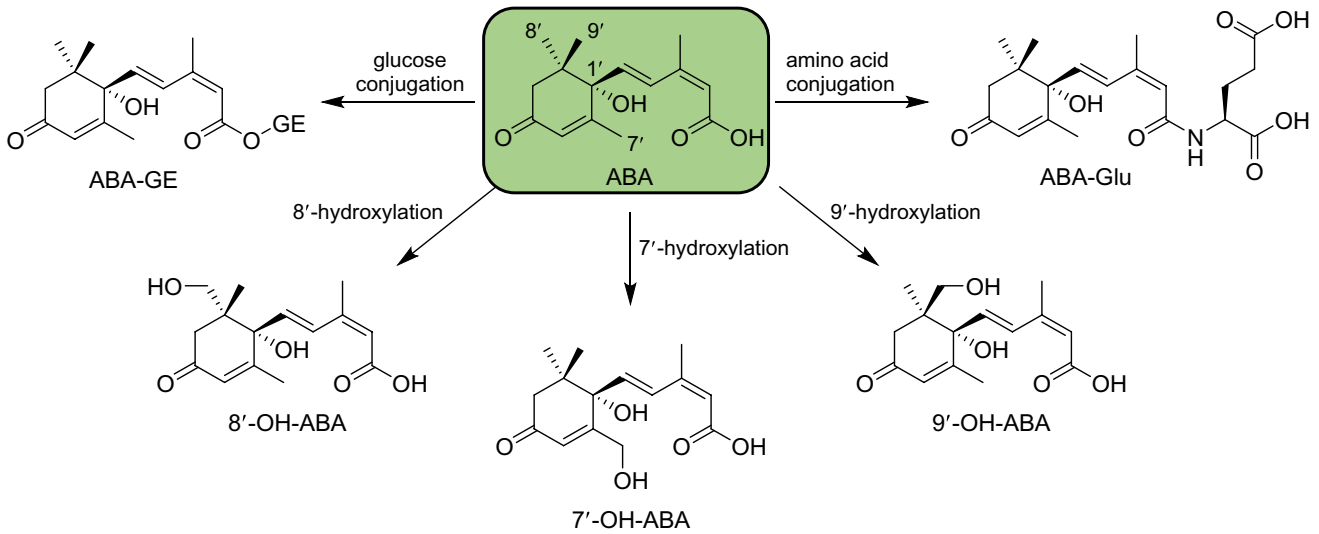


Fig. 1 The catabolic pathway of ABA in plants

quantitative analysis by LC−MS requires extensive sample purification to separate them from complex plant matrices. Extraction of analytes from different plant materials using water-miscible organic solvents, such as methanol, ethanol or acetone (Bai et al. 2018; Gomez-Cadenas et al. 1996; Hogge et al. 1992; Okamoto et al. 1993; Ross et al. 2004; Turečková et al. 2009; Záveská Drábková et al. 2015; Zhou et al. 2003), water (Gómez-Cadenas et al. 2002; Hradecká et al. 2007) or isopropanol:water:glacial acetic acid (80:19:1, v/v/v) (Nguyen et al. 2016) is usually followed by solid-phase extraction (SPE) based on cation- and anion-exchange sorbents (Cao et al. 2016; Chiwocha et al. 2003; Dobrev et al. 2005; Prado et al. 2014; Turečková et al. 2009; Záveská Drábková et al. 2015; Zhou et al. 2003). The final step for isolation of compounds from plant extracts may also involve immunoaffinity chromatography (IAC) (Hradecká et al. 2007; Turečková et al. 2009). Currently, diverse techniques are employed to detect and quantify ABA and its metabolites in plant materials (Široká et al. 2022; Tarkowská et al. 2014; Vrobel and Tarkowski 2023; Wang et al. 2020).

In this paper, we report the development of a sensitive analytical method based on purification using a unique monoclonal antibody (mAb), raised against C1-ABA and characterised by ELISA, that binds naturally occurring ABA conjugates with amino acids and glucose. We also report the combination of IAC using this mAb with a sensitive UHPLC-MS/MS analytical method for quantification of ABA and conjugates in the presence of isotopically labelled internal standards (ISs). To demonstrate the potential of our technique, we isolated and identified the first ABA amino acid conjugate in seedlings of *Pisum sativum* L., *Lepidium sativum* L. and *Triticum aestivum* L.

Materials and Methods

Reagents and Chemicals

(+)-*cis,trans*-ABA, (+)-*cis,trans*-ABA methyl ester (ABAMe), (±)-abscisyl-β-D-glucosyl ester (ABA-GE), [(+)-4,5,8´,8´,8´-$^{2}H_5$-ABA-GE] (d_5-ABA-GE), and [(+)-3´,5´,5´,7´,7´,7´-$^{2}H_6$-ABA)] (d_6-(+)ABA) were generous gifts from Olchemim Ltd. (Czech Republic). [$^{13}C_4$,^{15}N]-Asp, [$^{13}C_5$,^{15}N]-Glu, and [$^{15}N_2$]-Trp were purchased from Cambridge Isotope Laboratories, Inc. and ABA was obtained from Olchemim. Unlabelled ABA amino acid conjugates (ABA-AAs) (ABA-Gly, ABA-Ala, ABA-Asp, ABA-Glu, ABA-Trp, ABA-Phe, ABA-Leu, ABA-Ile, ABA-Val, ABA-Tyr, ABA-Pro), labelled [$^{13}C_4$,^{15}N]-ABA-Asp, [$^{13}C_5$,^{15}N]-ABA-Glu, and [$^{15}N_2$]-ABA-Trp were synthesised as described previously (Todoroki et al. 2011) with minor modification of the ABA chloride preparation step according to a previously published procedure (Pařízková et al. 2021). All other chemicals were purchased from Lach-Ner (Czech Republic) or Merck (Germany).

Methyl esters from unlabelled and labelled ABA-AA standards were prepared by methylation with an ethereal solution of diazomethane, as described in (Hradecká et al. 2007).

Methanol (MeOH, HPLC grade) and acetic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA), formic acid for LC–MS was obtained from VWR (Leuven, Belgium), and Oasis® HLB cartridges (60 mg, 3 mL) were from Waters (Milford, USA). The PBS buffer used contained 7.8 g of $NaH_2PO_4.2H_2O$ and 0.877 g of NaCl per litre and was adjusted to pH 7.2 using 0.1 M NaOH. Deionised (Milli-Q) water obtained from a Simplicity®UV water purification system (Darmstadt, Germany) was used to prepare all aqueous solutions. All other chemicals (analytical grade or higher purity) used in the LC–MS analysis were from BiosolveChimie (Dieuze, France).

Plant Materials

Nicotiana tabacum L. leaves were used for method validation. Plants were cultivated in a greenhouse under long-day conditions (16 h light, 22 °C/8 h dark, 18 °C cycle) for 8 weeks. Plant tissues were harvested after 24, 48, 72 and 96 h of water stress, immediately frozen in liquid nitrogen and then stored at − 80 °C until extraction and analysis.

Arabidopsis thaliana (ecotype Columbia, WT) seedlings were grown vertically in vitro in Petri dishes containing Murashige and Skoog medium in a growth chamber under long-day conditions (16 h light, 21 °C/ 8 h dark 21 °C cycle) for 10 days. Ten-day-old seedlings were harvested and immediately plunged into liquid nitrogen and stored at − 80 °C until extraction and analysis.

Pisum sativum L. and *Lepidium sativum* L. seedlings were grown in a desiccator at room temperature and harvested after 18, 24, 36 and 48 h of germination, immediately frozen in liquid nitrogen and then stored at − 80 °C until extraction and analysis.

Triticum aestivum L. seeds were left germinating in the cultivation room (16-h light/8-h dark) at 24 °C and harvested after 18, 24 and 48 h germination, immediately frozen in liquid nitrogen and then stored at − 80 °C until extraction and analysis.

Preparation and Characterisation of Anti-ABA Monoclonal Antibodies

(+)-*cis,trans*-Abscisic acid (ABA) was coupled through C1 to the tested proteins (bovine serum albumin-BSA, alkaline phosphatase-AP) by modification of the

carbodiimide-mediated cross-linking method (Weiler 1980). The immunogen (ABA-C_1-BSA) was used for immunisation of BALB/c mice as described previously (Oklestkova et al. 2017). Cell fusion and hybridomas were prepared according to (Harlow and Lane 1988). Several production hybridoma clones were selected and characterised, and based on its properties, the 1F5/G5 clone was selected for further experiments and to produce mAbs in CELLine bioreactor flasks (WHEATON®, Merck). mAbs were isolated from culture media using ammonium sulphate precipitation (Grodzki and Berenstein 2010). The affinity and specificity of the antibodies were characterised by a direct competitive ELISA test using a modified protocol (Oklestkova et al. 2017).

The authors declare that all institutional and national guidelines for the care and use of laboratory animals were followed.

Preparation and Use of Immunoaffinity Columns

The purified anti-ABA mAbs were coupled to *N*-hydroxysuccinimide ester-activated agarose (32 mg IgG/mL Affi-Gel 10, Bio-Rad, USA). Immunoaffinity (IA) columns were prepared by dispensing 0.5 mL of the immunoaffinity gel (IAG) into 3 mL polypropylene columns that were stored in PBS buffer (50 mM NaH_2PO_4, 15 mM NaCl, 0.1% NaN_3, pH 7.2) at 4 °C. The capacity and analyte recovery of the immunoaffinity gel was determined using standard solutions containing: (1) 12 methylated abscisates (ABA amino acid conjugates and ABA) and ABA-GE standards (ranging from 5 to 1000 pmol) or (2) 11 unmethylated ABA-AAs (ranging from 5 to 500 pmol) in methanol:PBS buffer (5:95, v/v). Solutions at each analyte concentration were prepared in quadruplicate and the whole experiment was repeated twice.

Extraction and Purification of ABA-GE, ABA and Its Amino Acid Conjugates

Different plant materials were frozen in liquid nitrogen and then ground into a fine powder using a mortar and pestle. Aliquots of 10–20 mg FW were weighed into 2-mL centrifuge tubes (Eppendorf, Germany), then 1 mL of ice-cold extraction solvent methanol/water/acetic acid (10/89/1, v/v/v) and 2 mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) were added, followed by homogenisation using a MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany). An IS mixture containing 20 pmol each of [$^2H_5$]-ABA-GE, [$^2H_6$]-ABA, [$^{13}C_4$,^{15}N]-ABA-Asp, [$^{13}C_5$,^{15}N]-ABA-Glu, and [$^{15}N_2$]-ABA-Trp was also added to the samples. The homogenates were centrifuged (20 000 rpm, 10 min, 4 °C, Hermle Z36 HK, Labortechnic GmbH, Germany) after 1 h of extraction, and the resulting pellets were re-extracted in the same way for 30 min. The combined extracts were purified using a Visiprep™ solid-phase extraction (SPE) vacuum manifold (Supelco®, Bellefonte, PA, USA) and 3 mL Oasis® HLB cartridges (60 mg, Waters, Milford, MA, USA) that had been conditioned with 2 mL methanol and equilibrated with 2 mL methanol/water/acetic acid (10/89/1, v/v/v). The samples (approx. 1.5 mL) were loaded onto the cartridges, which were washed with 1-mL methanol/water/acetic acid (10/89/1, v/v/v) and then eluted with 3 mL methanol/water/acetic acid (80/19/1, v/v/v). The eluates were dried under vacuum at 37 °C (CentriVap® Acid-Resistant, Labconco Corporation, USA). Subsequently, the evaporated samples were methylated with ethereal diazomethane (300 µl, 10 min, room temperature), then purified with a pre-immune column, processed by (+)-ABA-specific IAC as previously described (Hradecká et al. 2007), and finally analysed by UHPLC-MS/MS.

Method Validation

To validate the method, three sets of samples were prepared with four replicates and then analysed by UHPLC-MS/MS. The extraction and purification protocol described above was used for all samples. In the first set, 20 mg FW of 48 h water-stressed *N. tabacum* L. leaves were extracted in acidified 10% MeOH and then spiked before purification with 10 pmol of the corresponding IS. In the second set, identical plant tissue extracts were spiked before purification with a standard mixture containing 0.1, 1 or 10 pmol of nonlabelled standards for all the studied analytes and 10 pmol of the corresponding IS. The concentration of targeted nonlabelled analytes in the plant extract after purification was calculated by subtraction of their endogenous levels (obtained from the first set). Subsequently, the obtained results were compared with the known quantities of standards spiked into the second set of samples. The results of this comparison were considered to represent the method accuracy. In the third set, the same methods as in set 1 and 2 were used with extraction solvent (acidified 10% MeOH) without plant material. The method precision was calculated as the relative standard deviation (%) of analyte levels in the spiked samples.

Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)

An Acquity UPLC™ system (Waters, Milford, MA, USA) coupled to a Xevo TQ tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray (ESI) was used for analysis of nonlabelled and stable isotope-labelled ABA metabolites. The dried samples, after purification by IAC, were reconstituted in 40 µL of mobile phase (initial conditions) and then 15 µL of each sample was injected onto a Waters ACQUITY UHPLC BEH C18 (2.1 × 150 mm, 1.7 µm) column with a flow rate of 0.2 mL/

min at 40 °C. The analytes were separated by a linear gradient of 0–19 min to 35–60% A and 19.00–19.50 min to 60–100% A of MeOH (A) and 10 mM FA (B). Finally, the column was washed with 100% methanol for 3 min and equilibrated to the initial conditions (35:65 A:B, v/v) for 3.5 min. The MS settings were as follows: capillary voltage 0.75 kV; cone voltage 20–30 V; collision energy 13–22 eV; source/desolvation temperature 120/550 °C; and cone/desolvation gas flow, 70/650 $L.h^{-1}$. The dwell time of each MRM channel was calculated to provide 16 scan points per peak, with an inter-channel delay of 0.1 s. The retention time and parameters for MRM of separated compounds are listed in Table 1. Data acquisition and analysis were performed using the MassLynx™ software (version 4.2, Waters).

Results and Discussion

Anti-ABA Antibody Characteristics

Monoclonal antibodies were raised against (+)-*cis,trans*-ABA conjugated to BSA through its carboxyl group (C1) by modification of the carbodiimide-mediated cross-linker method (Hermanson 2008). Afterwards, mice were immunised with the ABA-C1-BSA conjugate to obtain spleen cells fused with myeloma cells. The growing hybridoma cells were tested for the production of mAbs specific for the ABA-BSA antigen using an enzyme-linked immunosorbent assay (ELISA) and those showing the best results were subsequently cloned by the limited dilution method (Eyer and Franek 2012). Several hybridoma clones producing anti-ABA mAbs were selected, of which clone ABA $1F_5/G_5$ was chosen as the best one for the production of a high affinity mAb. The latter mAb was further characterised by an enzyme-linked immunosorbent assay (ELISA). Figure 2 shows a typical standard curve for (+)-*cis,trans*-ABAMe together with the corresponding linearised log/logit plot (inset), which suggested a measuring range between 1.11×10^{-6} and 4.57×10^{-9} mol/L. The ELISA assay was very sensitive, enabling the detection of 4.57×10^{-9} mol/L for (+)-*cis,trans*-ABAMe. The mid-range (amount of antigen required for 50% inhibition) value was around 1.4×10^{-7} mol/L for this compound.

The specificity of the selected mAb was determined by cross-reactivity studies (Table 2). The mAb reacted most strongly with ABAMe (100%) and very well with (±)-*cis,trans*-ABA glucosyl ester (98%), whereas natural (+)-*cis,trans*-ABA (13%) was much less immunoreactive. Cross-reactivity with ABA-catabolites such as (−)-PA, (−)-DPA, (+)-neoPA, (±)-7´-OH-ABA and its methylated forms was negligible. On the other hand, the antibody interacted with all ABA conjugates bound via the C1 carboxyl group. Other authors have reported similar results for the specificity of anti-ABA-C_1 antibodies prepared using similar protein antigens (le Page-Degivry et al. 1984; Leroux et al. 1985; Rosher et al. 1985; Weiler 1979, 1980). However, in

Table 1 Optimised UHPLC–MS/MS parameters for the quantification of ABA and its conjugates. Values are means ± SD ($n = 22$)

Time window	Compound	Retention time (min)	MRM transition	Cone voltage (V)	Collision energy (eV)	LOD (pmol)	LOQ (pmol)	R^2
I	ABA-GE	4.62 ± 0.01	449.0 > 287.1	30	20	0.02	0.07	0.9990
	D_5ABA-GE	4.55 ± 0.01	454.0 > 292.1					
II	ABA-GlyMe	6.90 ± 0.01	318.0 > 229.0	25	16	0.03	0.10	0.9991
III	ABA-AlaMe	8.54 ± 0.01	332.0 > 229.0	25	18	0.02	0.07	0.9996
	ABA-$AspMe_2$	9.18 ± 0.02	390.0 > 229.0	25	18	0.01	0.05	0.9998
	ABA-$AspMe_2$-$^{15}N^{13}C_4$	9.17 ± 0.01	395.0 > 229.0					
	ABA-ProMe	9.79 ± 0.01	358.0 > 229.0	25	22	0.02	0.07	0.9996
	ABA-$GluMe_2$	10.17 ± 0.01	404.0 > 229.0	25	20	0.01	0.04	0.9999
	ABA-$GluMe_2$-$^{15}N^{13}C_5$	10.15 ± 0.01	410.0 > 229.0					
IV	ABA-TyrMe	12.94 ± 0.04	424.0 > 229.0	20	22	0.02	0.06	0.9970
	ABAMe	13.15 ± 0.01	261.0 > 191.1	24	13	0.01	0.03	0.9970
	D_6ABAMe	13.03 ± 0.01	267.1 > 191.1					
	ABA-ValMe	13.86 ± 0.01	360.0 > 229.0	25	20	0.02	0.06	0.9986
V	ABA-IIeMe	17.25 ± 0.01	374.0 > 229.0	25	20	0.02	0.06	0.9997
	ABA-LeuMe	17.61 ± 0.07	374.0 > 229.0	25	20	0.02	0.08	0.9995
	ABA-PheMe	18.33 ± 0.01	408.0 > 229.0	25	20	0.02	0.08	0.9995
	ABA-TrpMe	19.11 ± 0.01	447.0 > 229.0	25	20	0.03	0.09	0.9990
	ABA-TrpMe-$^{15}N_2$	19.11 ± 0.01	449.0 > 229.0					

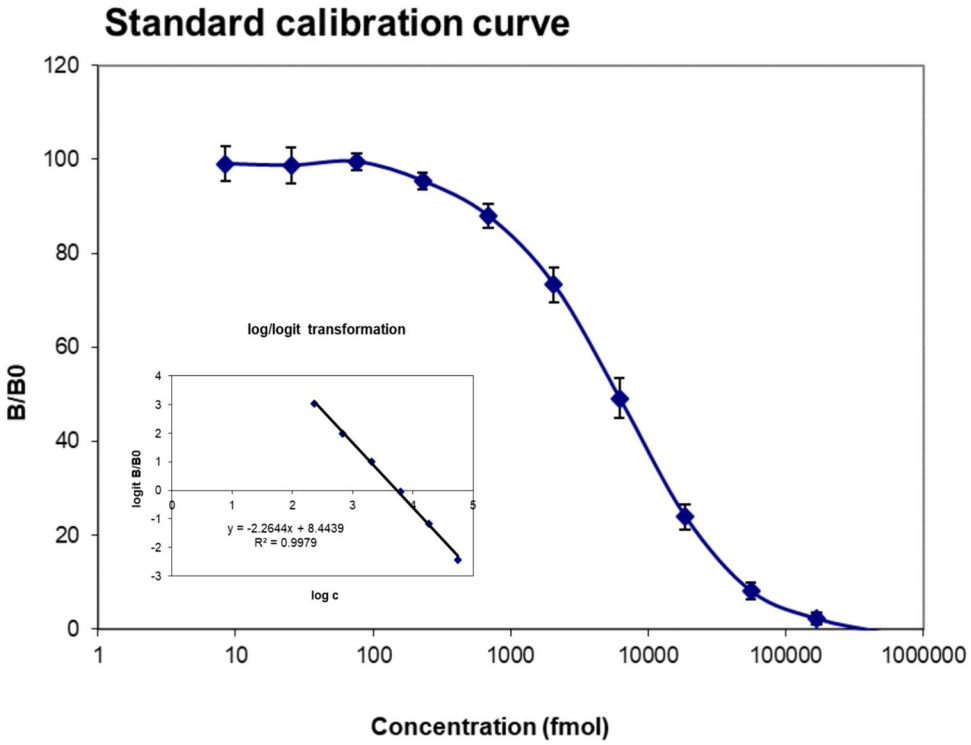


Fig. 2 Standard calibration curve for the (+)-*cis,trans*-ABAMe standard. The inset shows the linearised log/logit plot of the same data

Table 2 Molar cross-reactivities of ABA and structurally related compounds with monoclonal antibody ABA 1F5/G5

	Cross-reactivity (%)
(+)- *cis,trans*-ABAMe	100
(+)-*cis*,*trans*-Abscisic acid	12.6
(±)-*cis*,*trans*-Abscisic acid glucosyl ester	98
(±)-*cis*,*trans*-Abscisic acid alcohol	17.6
(−)-Dihydrophaseic acid methyl ester	< 0.01
(−)-Phaseic acid methyl ester	< 0.01
(+)-Neophaseic acid methyl ester	< 0.01
(+)-*cis*,*trans*-9´-OH-ABA methyl ester	0.33
(±)-*cis*,*trans*-7´-OH-ABA methyl ester	< 0.01
(+)-*cis*,*trans*-ABA-Alanine methyl ester	36.6
(+)-*cis*,*trans*-ABA-Glutamic acid dimethyl ester	143
(+)-*cis*,*trans*-ABA-Leucine methyl ester	91.2
(+)-*cis*,*trans*-ABA-Isoleucine methyl ester	106
(+)-*cis*,*trans*-ABA-Tyrosine methyl ester	323
(+)-*cis*,*trans*-ABA-Glycine methyl ester	37.5
(+)-*cis*,*trans*-ABA-Proline methyl ester	24.1
(+)-*cis*,*trans*-ABA-Valine methyl ester	44.8
(+)-*cis*,*trans*-ABA-Aspartic acid dimethyl ester	377
(+)-*cis*,*trans*-ABA-Tryptophan methyl ester	1140
(+)-*cis*,*trans*-ABA-Phenylalanine methyl ester	298

contrast to previous studies, we also tested the cross-reactivity with ABA amino acid conjugates and their methylated derivatives. All methylated conjugates were strongly immunoreactive, whereas non-methylated derivatives were less reactive (Table S1). Methylation of standards and samples in our case led to an increase in the sensitivity of the ELISA test. This enhancement was even more pronounced when used in IAC (see below). Our results were very similar to those obtained in previous immunological studies of ABA, indole-3-acetic acid and gibberellins, in which carboxyl-linked haptens also yielded antibodies that did not bind the free carboxylate well (Atzorn and Weiler 1983; Hradecká et al. 2007; Pěnčík et al. 2009; Turečková et al. 2009; Weiler et al. 1981). Owing to their high reactivity with ABA C1 conjugates, such antibodies are extremely useful for the immunoaffinity purification of diverse ABA metabolites.

Immunoaffinity Chromatography (IAC)

IAC is based on the reversible and selective interaction of an antigen with corresponding antibodies (preferentially IgG) immobilised on an appropriate support, such as Sepharose 4B or Affi-Gel 10® (Davis et al. 1985). IAC-based analytical protocols take advantage of the high selectivity of specific antibodies combined with the high resolution of separation techniques (Theodoridis et al. 2002). To remove substances that could exhibit non-specific interactions with Affi-Gel before passage through the immunoaffinity column, a precolumn containing 0.5 mL of IgG immobilised on Affi-Gel is used. The most important IAC parameter is capacity. When IAC columns are overloaded, recoveries fluctuate and the

Table 3 Test of capacity of monoclonal antibody with 0.5 mL of immunoaffinity gel for 5–1000 pmol of (A) (+)-*cis,trans*-abscisic acid methyl ester (ABAMe) and (B) (+)-*cis,trans*-abscisic acid (ABA)

(A)					
ABAMe (pmol)	5	10	50	100	1000
Gel capacity (pmol/0.5 mL)	4.77	8.92	26.64	35.59	45.99
Recovery (%)	95.4	89.2	53.28	35.59	4.60
SD (%)	0.11	0.33	1.64	4.06	2.83
(B)					
ABA (pmol)	5	25	100	500	1000
Gel capacity (pmol/0.5 mL)	0.03	0.1	0.4	1.9	1.5
Recovery (%)	0.58	0.52	0.37	0.38	0.15
SD (%)	0.0	0.0	0.1	0.3	0.4

calibration range becomes no longer linear. The sorbent capacity is defined as the maximum amount of an analyte that can be bound by a given volume of immunosorbent (Delaunay-Bertoncini and Hennion 2004; Hennion and Pichon 2003). In this paper, we describe development of an IAC method that is highly specific for ABA C1 conjugates based on a generic monoclonal anti-ABA antibody. Owing to cross-reactivity of the monoclonal mAb $1F_5/G_5$ antibody, the ability of the immunoaffinity sorbent (prepared by coupling the antibody to Affi-Gel 10) to trap ABA conjugates bound via the carboxyl group was examined. In the immunosorption of small molecules, the binding efficiency and capacity depend mainly on the density of the antibody immobilised on the support and the volume of IAG. Therefore, antibody concentrations recommended by the manufacturer (Bio-Rad Labs.) or reported by other authors (32 mg mL^{-1} gel) were used in this study (Davis et al. 1986).

The dynamic column capacity of the immunosorbent comprising 0.5 mL of IAG was determined using ABA and ABAMe standards with concentrations ranging from 5 to 1000 pmol, as described in Turečková et al. (2009). The binding and recovery capacities were determined by UHPLC-MS/MS (Table 3). The capacity of IAC for (+)-*cis,trans*-ABAMe was about 0.1 nmol/mL, whereas for (+)-*cis,trans*-ABA, it was negligible. IAC recoveries greater than 89% were obtained for ABAMe in the range 5 to 10 pmol and the recovery remained higher than 50% up to 50 pmol. After exceeding this amount, the immunoextraction recovery decreased rapidly. The capacity of the mAb-based IAG did not exceed the capacity of IAG prepared with polyclonal antibodies described previously (Hradecká et al. 2007; Turečková et al. 2009). Turečková et al. (2009) reported that the binding capacity of an IAG comprising a mixture of C1ABA1 and C1ABA2 polyclonal antibodies with high affinity was significantly lower after application of a mixture of ABA standards than in the presence of a single free competitor. When using IAC for purifying plant extracts, in which several cross-reacting catabolites are often present, the capacity and recovery must remain high for each of the target catabolites. Since our cross-reactivity study only investigated antibody binding parameters in the presence of a single free competitor, we used IAC-UHPLC-ESI-MS/MS to determine the extent of cross-reactivity when the immobilised antibody was exposed to a mixture of potentially cross-reacting compounds.

To test the capacity of the IAG to bind different ABA conjugates, as well as the effects on capacity of competition between ABA conjugates with different relative affinities, the following mixtures were applied and the constituent analyte recoveries determined by UHPLC-MS/MS: (1) a mixture of ABA and 12 conjugates and (2) a mixture of ABAMe, ABA-GE and 11 methylated ABA amino acid conjugates (ABA-AAMe) (Fig. 3, Fig. S1). Recoveries higher than 70% were obtained for most ABA-AAMe conjugates in the range 5 to 10 pmol, and the recoveries remained higher than 30% up to 100 pmol for seven methylated ABA conjugates (ABA-$GluMe_2$, ABA-ValMe, ABA-TrpMe, ABA-TyrMe, ABA-LeuMe, ABA-IleMe, and ABA-PheMe). However, the recoveries of all ABA-AAMe conjugates declined rapidly beyond this limit.

To compare the binding capacity of the mAb-based IAG, the binding capacity of a polyclonal antibody-based IAG described earlier by Hradecká et al. (2007) and Turečková et al. (2009) was also tested. Solutions containing mixtures of unmethylated and methylated ABA acids and their conjugates at levels ranging from 5 to 2500 pmol were purified using 0.5 mL of IAG and the resulting eluates were analysed by UHPLC-MS/MS. ABAMe and ABA-AAMe conjugates were found to be retained with high recoveries over the full concentration range tested (Fig. S2A), whereas the recoveries of ABA and unmethylated ABA-AAs were substantially lower (Fig. S2B). To increase the affinity for the IAG, it was important to convert the free acids to their methyl esters.

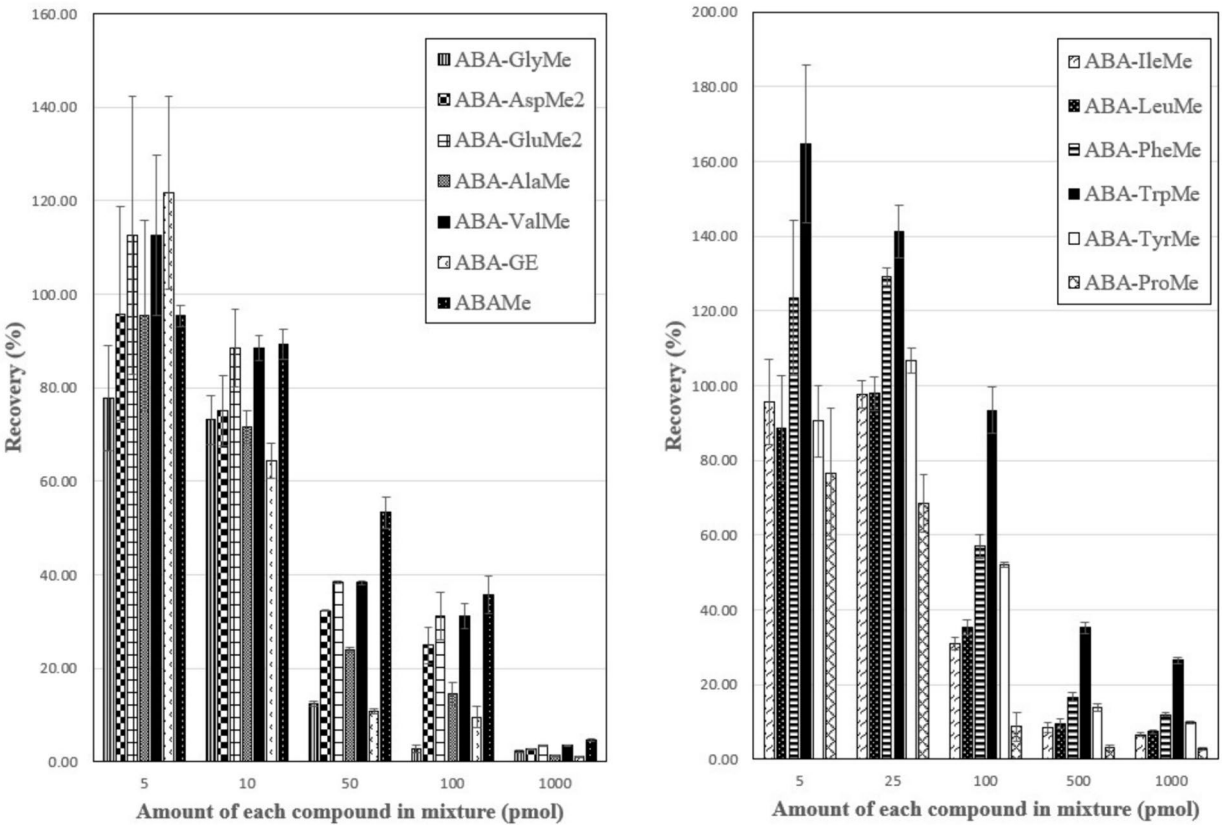


Fig. 3 Results of a binding capability test of the ABA $1F_5/G_5$ antibody with 0.5 mL of immunoaffinity gel for a mixture of ABAMe, ABA-GE and 11 ABA-AAMe conjugates

Hence, it was necessary to treat samples with diazomethane prior to immuno-purification, similarly to procedures published earlier (Hradecká et al. 2007; Pěnčík et al. 2009; Turečková et al. 2009).

The results of these experiments indicated that the maximum capacity of the IAG for ABA and its conjugates, and their recoveries, differed depending on the presence of other ABA-type ligands in the natural samples. On the other hand, at all concentrations tested, the differences in individual conjugate capacities did not fully correlate to the cross-reactivity data obtained by ELISA. Hence, the binding capacity of the antibodies appeared to be influenced by their immobilisation to varying degrees, depending on the metabolite. The cross-reactivity parameters for a complex mixture may differ from those of the primary antigen for a number of reasons, e.g. (1) differences in the K_a values of antibodies for cross-reacting substance in comparison to the primary antigen, (2) differences in the behaviour of immobilised antibodies on a gel, and (3) differences in the concentrations (relative and absolute) of individual cross-reacting substances in real samples (Nicander et al. 1993). The binding capacities were also probably influenced by the high availability of free antibody binding sites at the low tested concentrations. However, despite the slightly better properties of polyclonal antibodies compared to newly prepared mAbs, the polyclonal-based IAG is not used in routine purifications due to the rapid depletion of antibody stock obtained from animal sera and faster degeneration of IA columns. It is also always a challenge to obtain polyclonal antibodies with the same affinity and specificity characteristics. For this reason, the use of hybridomas producing mAbs is preferred. Furthermore, if desired, a homogenous population of antibodies with reproducible affinity and specificity can be obtained and produced continuously (Kannangara et al. 1989).

In summary, the prepared IAG was capable of satisfactorily binding methylated ABA and its conjugates. Levels of ABA and its known catabolites are typically found in plants at very low concentrations (pmol g^{-1} fresh weight) and its unknown amino conjugates are expected to be present at even lower concentrations (Dong et al. 2015). Due to the low mass (10–20 mg) of plant material required and high sensitivity of UHPLC-MS/MS for analysis, the binding capacity of the IAG was considered sufficient and the approach deemed applicable for the purification and isolation of ABA

amino conjugates from various plant species. In the future, it may be possible to develop more sensitive UHPLC-MS/MS approaches that allow the quantification and identification of unknown ABA-AAs from minute amounts (1–2 mg) of plant tissue.

We also demonstrated the benefits of using IAC for purifying ABA from plant tissue extracts. Immunoaffinity purification (by reducing matrix interferences) has been shown to significantly increase the selectivity and sensitivity (by more than 10 fold) of subsequent LC-ESI-MS analysis (Hradecká et al. 2007; Turečková et al. 2009). Similar results have also been observed for other phytohormones, i.e. the use of IAC before UHPLC-MS/MS helped to eliminate matrix effects and increase sensitivity (Eyer et al. 2016; Hauserová et al. 2005; Novák et al. 2008; Oklestkova et al. 2017). In the present study, we successfully used immunoaffinity extraction based on the mAb to isolate and quantify ABA and its conjugate with glucose (ABA-GE). However, unfortunately, this method cannot be used to isolate unmethylated ABA metabolites, such as DPA, neoPA, 7´-OH-ABA and ABA-AAs, due to their low affinity for the IAG. On the other hand, for the first time, we were able to isolate and identify a new naturally occurring ABA conjugate with glutamic acid from plants, as shown below.

UHPLC-MS/MS Method

Baseline separation of ABA-GE, ABAMe, and 11 ABA-AAMe conjugates was achieved using a linear gradient of 100% MeOH and 10 mM HCOOH as solvents A and B (Fig. 4). A mixture of 13 standards was separated on a reversed-phase column (2.1 × 150 mm, 1.7 µm) with the flow rate set to 0.2 mL/min and the column temperature set to 40 °C. The total run time of the procedure, including equilibration to initial conditions, was 26 min. The whole series of standards was eluted in 15 min, from ABA-GE at 4.62 ± 0.01 min to ABA-TrpMe at 19.11 ± 0.01 min. The retention times of all methylated standards of interest are listed in Table 1. The chosen chromatographic conditions allowed the chromatographic runs to be split into five targeted scan windows to obtain the greatest possible MS signal intensity for ABA-GE and all methylated compounds: 4.0–5.0 min, 5.50–7.30 min, 8.00–10.40 min, 12.45–14.30 min, and 16.70–19.60 min. The positive electrospray ionisation [ESI(+)] mode was used for determination of methylated ABA and its 12 conjugates, providing a sensitivity comparable with previously published data (Hradecká et al. 2007; Novák et al. 2012; Pěnčík et al. 2009; Turečková et al. 2009). The most prominent ions were selected as precursors, i.e. $[M+Na]^+$ for ABA-GE and $[M+H-H_2O]^+$ for ABAMe and ABA-AAMe conjugates. Finally, we investigated the effects of various parameters

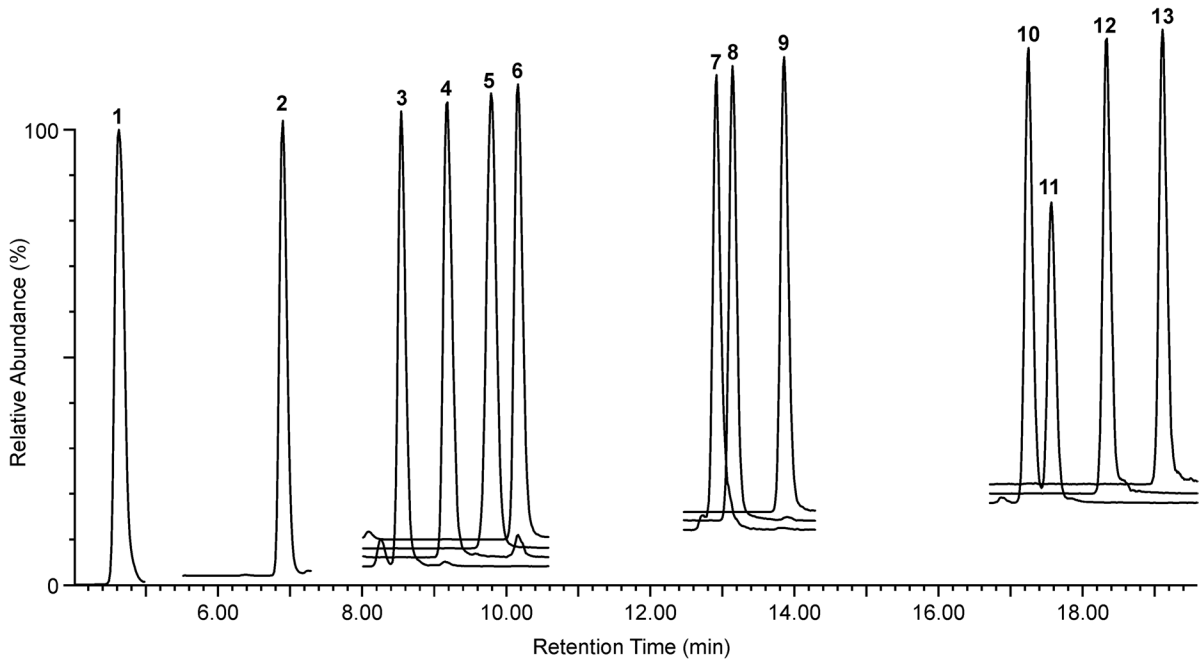


Fig. 4 Chromatographic separation of methylated ABA and its conjugate standards by UHPLC–ESI–MS/MS. The figure shows the MRM sums for 13 analytes represented by 1.5 pmol of each compound per injection using an Acquity UHPLC BEH™ C18 2.1 × 150 mm, 1.7 µm column. 1 – ABA-GE, 2 – ABA-GlyMe, 3 – ABA-AlaMe, 4 – ABA-Asp-Me_2, 5 – ABA-ProMe, 6 – ABA-GluMe_2, 7 – ABA-TyrMe, 8 – ABAMe, 9 – ABA-ValMe, 10 – ABA-IleMe, 11 – ABA-LeuMe, 12 – ABA-PheMe, and 13 – ABA-TrpMe

(e.g. collision energy, capillary voltage, and cone voltage) on the sensitivity of mass spectrometric detection and optimal values were determined for the UHPLC-ESI-MS/MS method (Table 1). Although methylation is not a prerequisite for ABA catabolite analysis, the derivatisation of samples before IAC and/or LC-ESI-MS analysis improved not only the response to ABA and its conjugates but also the affinity and capacity of the IAC approach and ABA stability (the methyl ester of natural ABA is very stable against UV radiation (Hradecká et al. 2007)) in the extracts. The advantages of using derivatisation have been described in previous studies for ABA, as well as other plant hormones (Hradecká et al. 2007; Pěnčík et al. 2009; Turečková et al. 2009). Thus, methylation of samples and standards may help to improve the stability, extraction yield, recovery, sensitivity, and reliability in analyses of ABA and its conjugates. In the future, it would be advantageous to optimise the methylation process to use other derivatisation reagents that are safer, more accessible, and easier to handle than diazomethane, e.g. trimethylsilyl)-diazomethane (TMS-diazomethane; Verslues 2017; Todoroki et al. 2011).

Calibration curves were generated from analyses of 11 solutions with different amounts of each unlabelled analyte and known fixed amounts of an appropriate IS as follows: 0.0015, 0.0075, 0.015, 0.075, 0.15, 0.75, 1.5, 7.5, 15, 75 and 150 pmol/7.5 pmol (unlabelled standards/labelled IS/15 µl of injection). Hence, 11-point linear calibration curves were constructed for each target analyte based on the response areas for duplicate consecutive separate injections of diluted authentic standard solutions. The resulting data were plotted after logarithmic transformation. The concentrations of endogenous ABA, ABA-GE and 11 ABA-AAMe conjugates in the plant samples were determined from the area ratios of endogenous to corresponding stable isotope-labelled standards (Rittenberg and Foster 1940). As listed in Table 1, good linearity was obtained in the range 0.0075–150 pmol with linear correlation coefficients (R^2) greater than 0.997.

The limit of detection (LOD) and limit of quantitation (LOQ) are important parameters used to validate analytical methods. LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, whereas LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy (Araujo 2009). In the present study, LOD and LOQ were calculated based on the standard deviation of the response (s_b) of the calibration curve and the slope (k) of the regression line, i.e. LOD = 3 (s_b/k) and LOQ = 10 (s_b/k). The LOD and LOQ values for ABA and its conjugates are presented in Table 1.

Method Validation

To validate our newly developed method, levels of ABA and its conjugates were measured in four replicates of 20 mg FW of *N. tabacum* L. leaves from plants that had been water stressed for 48 h. The method precision and accuracy were calculated based on the standard addition method using extraction solvent (blank) and a purified extract of *N. tabacum* L. spiked with or without a mixture containing 0.1 pmol, 1 pmol or 10 pmol of nonlabelled standards for all the studied analytes (Matuszewski et al. 2003). 10 pmol of each IS ([$^{13}C_4$,^{15}N]-ABA-Asp, [$^{13}C_5$,^{15}N]-ABA-Glu, [$^{15}N_2$]-ABA-Trp, [$^{2}H_5$]-ABA-GE and [$^{2}H_6$]-ABA) was added to each sample before purification. The extraction solvent (matrix free) and plant extracts were purified by SPE (HLB cartridge), then methylated, and followed by (+)-ABA-specific IAC, as described above. The concentration of each analyte was calculated using the standard isotope dilution method and compared with the concentration of appropriate standard solution. The analytical accuracy (determined as percentage bias (% bias)) for 0.1 pmol (low), 1 pmol (medium) and 10 pmol (high) concentration in matrix samples ranged between − 3.36 and 18.65, − 8.12 and 47.72 and − 12.74 and 41.83, respectively (Table 4). The analytical precision ranged, on average, from 0.76 to 14.59% (Table 4). The spiked extraction solution samples yielded similar precision and accuracy results (Table 4). Overall, the results confirmed that the method could serve as a powerful, precise and accurate tool for the targeted profiling of ABA and its conjugates, meeting all bioanalytical validation parameters (Nováková, 2013). Our methodology presented here could be further improved by internal standardisation of some of the missing labelled ABA amino conjugates.

Quantification of ABA and Its Conjugates in Various Plant Material

Finally, the newly developed and validated mAb-based immuno-purification method coupled with sensitive MS/MS detection was applied for the determination of ABA and its conjugates in various plant species. In this study, four different plant species were used to estimate the endogenous levels of ABA and its conjugates.

In the first experiment, along with free ABA, its main glucosyl ester conjugate was detected in 10-day-old *A. thaliana* seedlings. Levels of ABA and ABA-GE were 37 ± 1.4 pmol/g FW and 47 ± 7 pmol/g FW, respectively. No ABA amino acid conjugates were detected.

Leaves from control and water-stressed tobacco (*N. tabacum* L.) plants were used for the next experiment. ABA is known to play a vital role in the response to abiotic stress, especially in water-deficient environments, where plant species increase cellular levels of ABA to close stomata (Ali

Table 4 Method validation. Analytical precision and accuracy of the whole procedure for different amounts of ABAMe, ABA-GE, and three ABA-AAMe

Compound	Determined spiked content								
	No matrix (10% MeOH; $MeOH/H_2O/CH_3COOH$; 10/89/1, v/v/v)								
	100 fmol	Analytical accuracy (% bias)	Method precision (% RSD)	1 pmol	Analytical accuracy (% bias)	Method precision (% RSD)	10 pmol	Analytical accuracy (% bias)	Method precision (% RSD)
A									
ABAMe	111.43 ± 10.42	11.43	9.35	1.13 ± 0.14	13.62	12.39	10.84 ± 1.28	8.39	11.81
ABA-GE	99.64 ± 12.87	− 0.36	12.92	1.06 ± 0.07	5.93	7.05	13.06 ± 0.48	30.61	3.65
ABA-$GluMe_2$	98.04 ± 5.42	− 1.96	5.53	0.93 ± 0.02	− 6.82	2.04	8.85 ± 0.13	− 11.46	1.47
ABA-TrpMe	151.39 ± 7.46	51.39	4.93	1.54 ± 0.03	53.86	1.69	14.37 ± 0.27	43.69	1.87
ABA-$AspMe_2$	116.14 ± 7.62	16.14	6.56	1.08 ± 0.09	8.21	8.15	11.44 ± 1.25	11.46	10.93
	Determined spiked content								
	Matrix (*Nicotiana tabacum* L. leaves)								
Compound	100 fmol	Analytical accuracy (% bias)	Method precision (% RSD)	1 pmol	Analytical accuracy (% bias)	Method precision (% RSD)	10 pmol	Analytical accuracy (% bias)	Method precision (% RSD)
B									
ABAMe	118.65 ± 11.00	18.65	9.27	0.98 ± 0.06	− 1.64	5.94	8.72 ± 1.28	− 12.74	14.59
ABA-GE	103.85 ± 10.68	3.85	10.29	1.12 ± 0.03	12.11	2.69	13.66 ± 0.57	36.63	4.16
ABA-$GluMe_2$	96.64 ± 1.71	− 3.36	1.77	0.92 ± 0.01	− 8.12	0.76	8.86 ± 0.05	− 11.14	0.61
ABA-TrpMe	112.76 ± 8.90	12.76	7.89	1.47 ± 0.03	47.72	1.88	14.18 ± 0.29	41.83	1.96
ABA-$AspMe_2$	103.54 ± 2.50	3.54	2.41	1.02 ± 0.03	1.52	2.94	10.10 ± 0.16	1.01	1.58

Extraction solvent (A) and extracts of 20 mg FW of 48-h water-stressed *Nicotiana tabacum* L. leaves (B) were spiked with 0.1, 1 or 10 pmol of authentic standards, then purified using the Oasis®HLB sorbent and immunoaffinity chromatography, and analyzed by UHPLC-ESI(+)-MS/MS. All experiments were performed four times

et al. 2020). Water stress-induced ABA accumulation is the result of a precise balance between its biosynthesis, catabolism, transport and reversible conjugation with glucose (Lee et al. 2006; Nambara and Marion-Poll 2005). Our results showed that when plants were exposed to water stress conditions, the concentration of endogenous ABA increased in stressed tobacco from 25 ± 5 to 2775 ± 114 pmol/g FW. Lee et al. (2006) and Han et al. (2020) have demonstrated that formation of free ABA in response to drought may be due to ABA-GE hydrolysis by AtBG1/BGLU18 since drought was found to induce the synthesis of β-glucosidases and activate the transport of ABA-GE into the endoplasmic reticulum (ER) from the apoplast or vacuoles. However, we did not observe a decrease in ABA-GE content in water stress-exposed tobacco plants but rather an increase accompanied by an increase in endogenous ABA (from 76 ± 3 to 2701 ± 131 pmol/g FW). An increase of ABA and ABA-GE content in response to water stress in different plant species has previously been described by several groups (López-Carbonell et al. 2009; Pashkovskiy et al. 2019; Thameur et al. 2014, 2011; Zandalinas et al. 2016). In the present study, no ABA amino acid conjugates were detected in control or stressed *N. tabacum* L. leaves.

In the final experiment, *P. sativum* L., *L. sativum* L., and *T. aestivum* L. seedlings were collected after 18, 24, 36 and 48 h of germination and then used for immuno-purification and subsequent UHPLC-MS/MS analysis. As shown in Fig. 5, the concentration of ABA and ABA-GE in *P. sativum* L. seedlings increased from 80 ± 7 to 333 ± 18 pmol/g FW and from 5 ± 0.2 to 20 ± 0.4 pmol/g FW, respectively, between 18 and 48 h of germination, whereas the content of ABA-Glu decreased from 30 ± 4 to 8 ± 0.5 pmol/g FW. Hypothetically, a decrease in ABA-Glu levels and an increase in ABA levels during germination may indicate that accumulated ABA-Glu was hydrolysed to release free active ABA, serving as a temporary form of ABA production, as has been described for other plant hormones (Hayashi et al. 2021; Ludwig-Müller 2011; Rampey et al. 2004). An earlier study by Todoroki et al. (2011) suggested that different plants may have hydrolysing activity towards different ABA-AAs and thus ABA-AAs may function as a species-selective pro-hormone for ABA. Further studies of ABA-AA conjugates are necessary to test this hypothesis. A similar

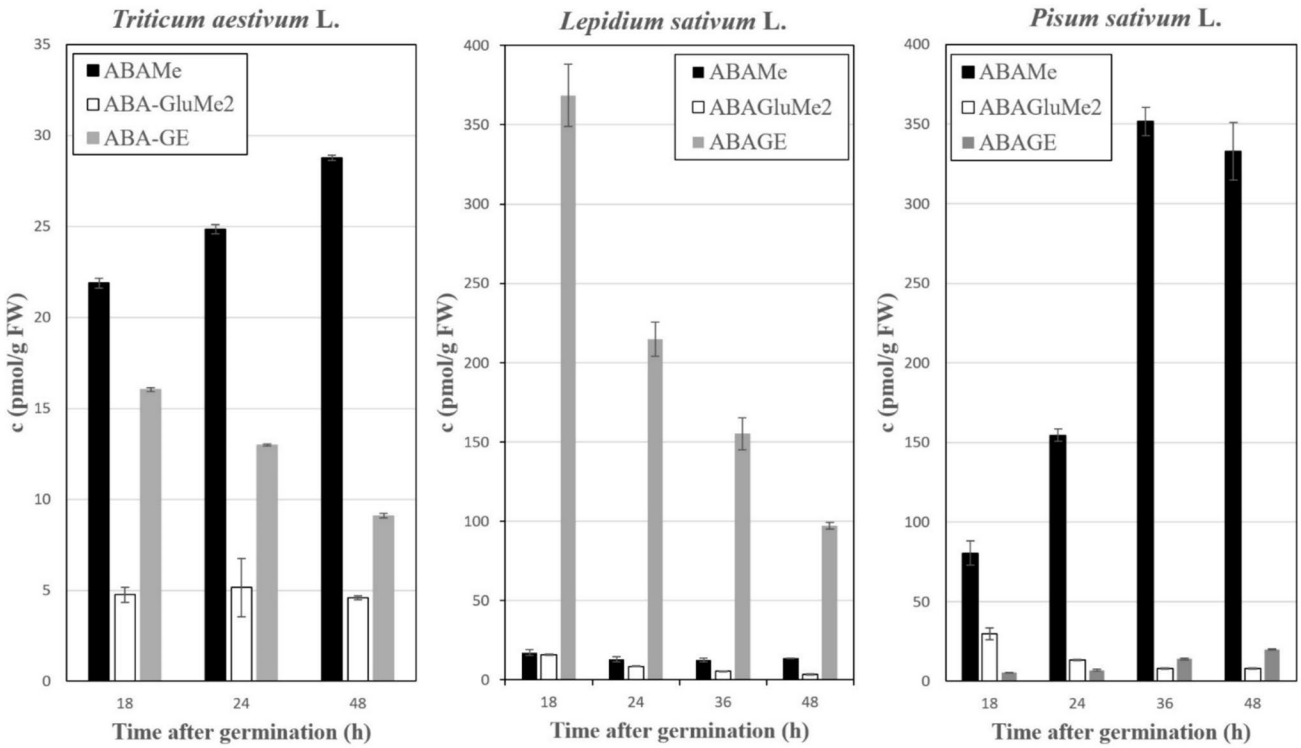


Fig. 5 Levels of ABA and its conjugates (ABA-GE, ABA-Glu) in *Triticum aestivum* L., *Pisum sativum* L., and *Lepidium sativum* L. seedlings collected after 18, 24, 36 and 48 h of germination (mean ± SE, $n = 4$)

trend for ABA-Glu was observed in germinating *L. sativum* L. and *T. aestivum* L. plants (Fig. 5). However, levels of ABA and ABA-GE in *L. sativum* L. and *T. aestivum* L. seedlings decreased, in contrast to *P. sativum* L. seedlings. Other ABA-AAs have not yet been identified in these types of plant tissue. The role of ABA-AAs, especially ABA-Glu, in the plant physiology of abscisates in monocots and dicots is still unclear.

In summary, our results suggested that ABA may be conjugated to amino acids in some plants. The new conjugation pathway was confirmed in young legume seedlings but not in ten-day-old *A. thaliana* seedlings and short-term water-stressed tobacco leaves. Given the observed decreasing levels of ABA-Glu during germination, we hypothesise that other unknown ABA-AAs may also be present, particularly in seeds. The localisation, identification and role of ABA-Glu and other unknown ABA-AAs in various seeds will be the subject of future biological studies.

Identification of a New ABA Amino Conjugate – ABA-L-Glutamate

Some phytohormones, such as indole-3-acetic acid (IAA), jasmonic acid (JA) and salicylic acid (SA), have been shown to occur conjugated to different amino acids. The role of amino-acid-conjugated phytohormones varies depending on the specific hormone and amino acid (Westfall et al. 2016, 2013). In the present work, identification of ABA-Glu analysed in *P. sativum* L., *L. sativum* L., and *T. aestivum* L. seedlings was based on immunoaffinity purification, which was developed to specifically interact with all ABA conjugates bound via the C1 carboxyl group. Pěnčík et al. (2009) also used immunoaffinity extraction followed by UHPLC-MS/MS analysis to isolate and identify new occurring IAA-AA conjugates with Gly, Phe and Val in *Helleborus niger* L. Similarly, Eyer et al. (2016) utilised IAC-UHPLC-MS/MS to confirm the presence of 2,4-D-amino acid conjugates (2,4-D-Asp and 2,4-D-Glu) in *Arabidopsis* seedlings. In Fig. 6, a chromatogram of the newly isolated ABA-Glu is compared with a chromatogram of the corresponding authentic compound after methylation. The retention time (10.15 min) of the peak for the compound isolated from *P. sativum* L. and *T. aestivum* L. seedlings collected 18 h after germination was practically identical to that of the synthetic ABA-GluMe$_2$ standard. Spiking of plant extract with synthetic standard of ABA-Glu at known concentrations (0, 0.1, 1, and 10.0 pmol) also confirmed the detection of chromatographic peak of the ABA-GluMe$_2$ (Fig. S3). Moreover, both chromatograms were also obtained in MRM mode, with peaks corresponding to the ABA-GluMe$_2$ $[M+H-H_2O]^+$ ion at *m/z* 404 and its fragment at *m/z* 229 after the loss of methylated glutamate. Finally, co-elution of a stable isotope-labelled standard

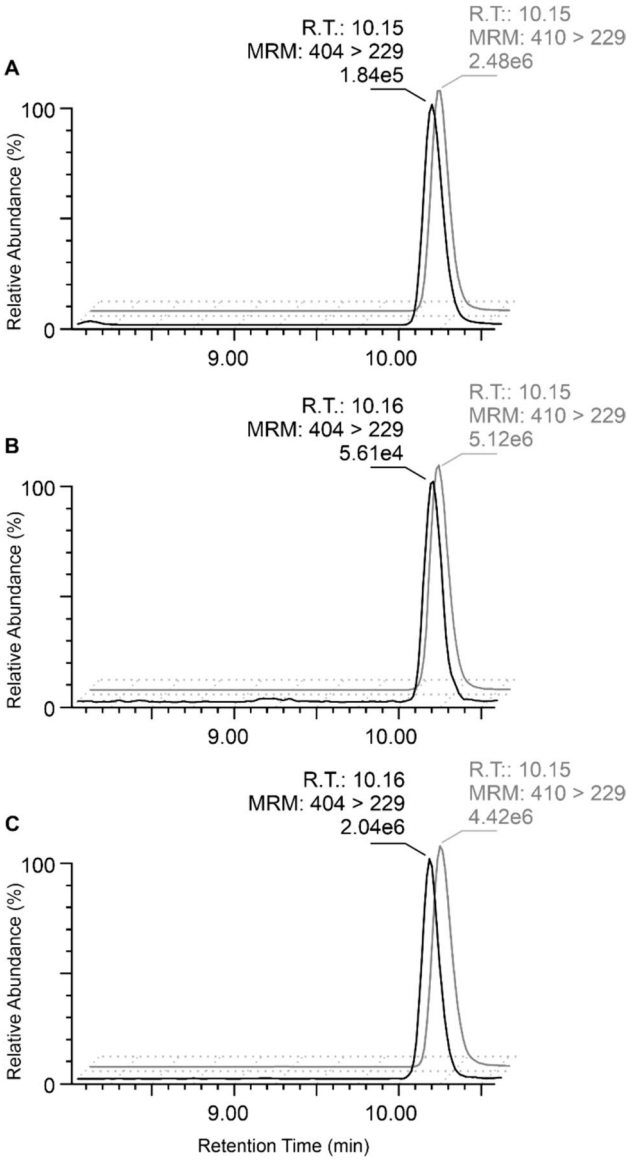


Fig. 6 MRM chromatogram of ABA-L-glutamate isolated from *Pisum sativum* L. (A) and *Triticum aestivum* L. (B) seedlings collected 18 h after inducing germination and chromatogram of the corresponding authentic compound (C) with internal standard of ABA-GluMe$_2$ ([$^{13}C_5$,^{15}N]-ABA-GluMe$_2$)

[$^{13}C_5$,^{15}N]-ABA-GluMe$_2$ with an endogenous compound also proved the presence of ABA-Glu in *P. sativum* L., *L. sativum* L. and *T. aestivum* L. seedlings (Fig. 6).

Conclusion

In the present study, new C1-abscisic acid (ABA)-specific mAbs were raised, characterised by ELISA and subsequently used for the preparation of an IAG. The immunoaffinity sorbent was successfully applied for selective isolation of endogenous C1-ABA conjugates, including ABA-GE, methylated ABA and 11 methylated amino acid ABA conjugates (ABA-AAMe) prior to their UHPLC-MS/MS analysis. Use of IAC-based purification helped to eliminate matrix effects and increase the sensitivity and selectivity of the method. Further, the method allowed the fast and easy isolation and subsequent identification of a novel naturally occurring ABA conjugate from plants, the first with an amino acid. Thus, a new ABA conjugation pathway was discovered, which was represented by ABA-L-glutamate detected in *P. sativum* L., *L. sativum* L. and *T. aestivum* L. seedlings. In contrast, no ABA-AAs were found in 10-day-old seedlings of *A. thaliana* or leaves of control or water-stressed *N. tabacum* L. plants. The discovery of ABA-L-glutamate provides an opportunity for investigation of a new regulatory mechanism of ABA metabolism. Further studies are also needed to determine the biological roles of ABA-AAs.

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Declarations

Competing Interest The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

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